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Role of DGKα and DGKζ in the control of lipid metabolism in breast cancer: Implications for therapeutic intervention

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Hope lies in dreams, in imagination and in the courage of those who dare to make dreams into reality.

Jonas Salk

INDEX

Abbreviations ..... **I**

Summary **1**

Resumen **5**

Introduction **9**

1. Breast cancer **11**

1.1. The normal mammary gland 11

1.2. Breast cancer: classification and clinical implications 11

2. Lipid metabolism in tumors **13**

2.1. Readjustment of lipid metabolism in tumors 13

2.2. Lipid biosynthesis 13

2.3. Transcriptional regulation of lipid biosynthesis 14

2.4. The role of DAG and PA as metabolic precursors 15

3. Lipid-mediated pathways in cancer **17**

3.1. The PI3K-Akt axis 17

3.1.1. PI3K-Akt axis alterations in breast cancer 18

3.2. The PA-mTOR axis: an explanation for rapamycin resistance 19

3.3. PA-mediated regulation of the Ras-ERK axis 20

3.4. The PKC-PKD axis in breast cancer 21

3.5. DAG/PA offers new opportunities for breast cancer treatment 23

4. Diacylglycerol kinases **23**

4.1. Classification 24

4.2. Structure 24

4.3. Diacylglycerol kinases functions 25

4.4. Diacylglycerol kinases and cancer 26

4.5. DGKα 26

4.5.1. Transcriptional regulation of the DGKα gene 26

4.5.2. Regulation of DGKα activity 27

4.5.3. DGKα functions 27

4.6. DGKζ 28
4.6.1. DGKζ expression 28
4.6.2. Regulation of DGKζ activity and subcellular localization 28
4.6.3. DGKζ functions 29

Objectives 31

Material and methods 35

Results 52

1. Analysis of the expression and activity of DGKα and DGKζ isoforms in breast cancer and the normal mammary gland and correlation with that of their regulators 54

1.1. Determination of DGK expression and activity in breast cancer cell lines 54

1.2. Analysis of DGKα expression in the mouse normal mammary gland 58

2. Contribution of DGKα and DGKζ to breast cancer progression 60

2.1. Consumption of DAG by DGKζ promotes SREBP-1 processing 60

2.2. Contribution of the DGK pathway to the maintenance of the oncogenic axes that promote breast cancer progression 68

2.3. Contribution of the DGK to the development of pharmacological resistance in breast cancer cells 76

3. Investigate the relevance of DGK expression and/or activity for breast cancer initiation and progression *in vivo* 80

4. Establish a reference framework to evaluate the potential of the DGK pathway as a target for therapeutic intervention 86

Discussion 89

1. Strong DGKα expression in untransformed mammary cells suggests a tumor suppressor role 91

2. DGKα and DGKζ contribute to the maintenance of lipid metabolism and oncogenic axes in breast cancer 94

2.1. DAG consumption helps to maintain the lipogenic metabolism of breast tumors 94

2.2. PA generation is needed for the maintenance of the PI3K-Akt-mTOR axis 98

3. DGK offers new opportunities for breast cancer treatment 103

Conclusions 107

Conclusiones 111

References 115

Agradecimientos..... 131

Appendix 134

ABBREVIATIONS

AC: adenocarcinoma
AF: Alexa Fluor
ALK: anaplastic lymphoma kinase
AMPK: AMP-activated protein kinase
APC-Cy7: tandem conjugate of allophycocyanin-cyanine
ATP: adenosine trisphosphate
CDC42: cell division control protein 42
CDP: cytidine diphosphate
CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CoA: coenzyme A
cDNA: coding deoxyribonucleic acid
Cyc: cyclin
DAB: 3,3'-diaminobenzidine
DAG: diacylglycerol
DAPI: 4',6-diamidino-2-phenylindole
DGK: diacylglycerol kinase
DMEM: Dulbecco's Modified Eagle Medium
ECM: extracellular matrix
EDTA: ethylene diamine tetraacetic acid
EGF: epidermal growth factor
EGFR: EGF receptor
EGTA: ethylene glycol tetraacetic acid
EpCAM: epithelial cell adhesion molecule
ER: estrogen receptor
ERK: extracellular signal-regulated kinase
FA: fatty acid(s)
FACS: fluorescence-activated cell sorting
FAK: focal adhesion kinase
FBS: fetal bovine serum
FFPE: formalin-fixed paraffin-embedded
FITC: fluorescein isothiocyanate
FRB: FKBP (FK506 binding protein)-rapamycin binding
GEF: guanine exchange factor
GPCR: G protein-coupled receptor

GPR30: G protein-coupled estrogen receptor 30
GTP: guanidine trisphosphate
Hepes: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2: human epidermal growth factor receptor-type2
HGF: hepatocyte growth factor
HR: hormone receptor
HRP: horseradish peroxidase
IDC: intraductal carcinoma
IL: interleukin
ILK: integrin-linked kinase
kDa: kilodalton
LEF-1: lymphoid enhancer-binding factor 1
MAPK: mitogen-activated protein kinase
MARCKS: myristoylated alanine-rich C-kinase substrate
MEM: minimum essential medium
MNR: magnetic nuclear resonance
NADPH: nicotinamide adenine dinucleotide phosphate hydrogen
NEAA: non-essential amino acids
NLS: nuclear localization sequence
PA: phosphatidic acid
PAGE: polyacrylamide gel electrophoresis
PAP: phosphatidic acid phosphohydrolase
PBS: phosphate buffer saline
PDK1: phosphoinositide-dependent protein kinase 1
PE: phycoerythrin
PE-Cy7: phycoerythrin-cyanine conjugate
PH: pleckstrin homology
PI3K: phosphatidylinositol-3-kinase
PIP5K: phosphatidylinositol-4-phosphate 5-kinase
PKB: protein kinase B
PKC: protein kinase C
PKD: protein kinase D
PLC: phospholipase C
PLD: phospholipase D

PR: progesterone receptor
PtdCho: phosphatidylcholine
PtdEtn: phosphatidylethanolamine
PtdIns: phosphatidylinositol
$PtdInsP_2$: phosphatidylinositol-4,5-bisphosphate
$PtdInsP_3$: phosphatidylinositol-3,4,5-trisphosphate
PtdSer: phosphatidylserine
PTEN: phosphatase and tensin homologue
PTP1B: protein tyrosine-phosphatase 1B
PTPI51: protein tyrosine-phosphatase-interacting protein 51
PVDF: polyvinylidene fluoride
RasGRP: Ras guanyl-nucleotide releasing protein
RB: retinoblastoma protein
RCP: Rab-coupling protein
RPMI: medium developed at the Roswell Park Memorial Institute
RTK: receptor tyrosine kinase
RT-PCR: retrotranscriptase polymerase chain reaction
SCAP: SREBP cleavage-activating protein
SDS: sodium dodecyl sulfate
SEM: standard error of the mean
SFK: Src-family kinases
sh-RNA: short-hairpin RNA
SIN: SAPK-interacting protein
SOS: son of sevenless
SREBP: sterol-regulatory element binding protein
TAG: triglyceride
TCA: tricarboxylic acid
TCR: T cell receptor
TN: triple negative
mTORC1: mammalian target of rapamycin complex 1
mTORC2: mammalian target of rapamycin complex 2
VEGF: vascular endothelial growth factor
WT: wild type

SUMMARY

Diacylglycerol kinases (DGK) are a family of lipid kinases that phosphorylate diacylglycerol (DAG) to produce phosphatidic acid (PA). DAG and PA are two lipids with functions as metabolic intermediates as well as second messengers, which allows integrated control of lipid metabolism with nutrient sensing and cell growth. Both lipids lie at the core of tumor-promoting axes, but their contribution to the transformed phenotype has not been thoroughly determined.

Our aim was to determine the input of DGK α and ζ isoforms to breast cancer progression and maintenance, to identify new therapeutic approaches for this disease. We found that the two isoforms differed in their expression patterns and function. *DGKα* gene expression correlated inversely with activation of the PI3K-Akt-FoxO pathway; thus, it was expressed strongly in untransformed breast-derived cell lines. In contrast, DGKζ expression was stronger in transformed cells.

DGKα and DGKζ used several mechanisms to promote breast cancer progression. DGKζ-dependent DAG consumption was necessary for the maintenance of lipid metabolism in breast tumors, via control of the SREBP-1 transcription factor. We found that DGK-generated PA was needed for the supply of substrates and for maintenance of phosphotyrosine signals that fuel the PI3K-Akt-mTOR axis. Moreover, we show that DGKα and DGKζ were upstream regulators of mTORC1, but only DGKα modulated Akt activation as well as that of the Src tyrosine kinase.

DGK inhibition reduced tumor growth *in vitro* and *in vivo*, without switching on other signaling pathways that could provide therapeutic resistance, such as the ERK or HIF-1α axes. Finally, we demonstrate that DGKα depletion in highly malignant breast cancer-derived cells impaired tumor development *in vivo*, as assessed in a mouse xenograft model.

Our data show important roles for the DGKα and DGKζ isoforms in promoting tumor progression, and suggest that the DGK pathway could represent an effective target for anti-cancer therapies.

RESUMEN

Las diacilglicerol quinasas (DGK) son una familia de lípido quinasas que fosforilan el diacilglicerol (DAG) para producir ácido fosfatídico (PA). El DAG y el PA son dos lípidos que tienen funciones como intermediarios metabólicos y segundos mensajeros, por lo que permiten el control integrado del metabolismo con la detección de nutrientes y el crecimiento celular. Ambos lípidos están en el núcleo de rutas oncogénicas, pero su contribución al fenotipo maligno no se ha determinado completamente.

Nuestro objetivo fue determinar la contribución de las isoformas DGKα y DGKζ a la progresión y mantenimiento del cáncer de mama con el fin de encontrar nuevas aproximaciones terapéuticas frente a esta enfermedad. Hemos encontrado que ambas isoformas difieren en su patrón de expresión y funcionalidad. La expresión de *DGKα* correlaciona inversamente con la activación del eje PI3K-Akt-FoxO, y por lo tanto, su expresión es elevada en líneas celulares de mama no transformadas. Su expresión, además, varía a lo largo del desarrollo de la glándula mamaria. La expresión de DGKζ, por el contrario, es más alta en células transformadas.

Las DGKα y DGKζ promueven la progresión del cáncer de mama a través de varios mecanismos. El consumo de DAG por la DGKζ es necesario para el mantenimiento del metabolismo lipídico de los tumores, mediante el control del factor de transcripción SREBP-1. Por su parte, el PA generado por DGK es necesario para proporcionar sustratos y mantener señales de fosfotirosina que activan el eje PI3K-Akt-mTOR. Sin embargo, aunque la DGKα y DGKζ regulan mTORC1, sólo la DGKα modula la activación de Akt y la tirosina quinasa Src.

La inhibición de DGK reduce el crecimiento tumoral *in vitro* e *in vivo* sin encender otras rutas de señalización que pueden proporcionar resistencia terapéutica, como los ejes de ERK o HIF-1α. Finalmente, demostramos que la disminución de DGKα en células de cáncer de mama altamente maligno impide su crecimiento *in vivo*, ensayado en un modelo de xenotransplante en ratón.

Nuestros datos otorgan funciones importantes a las isoformas DGKα y DGKζ en promover la progresión tumoral, y sugieren que la ruta de DGK puede representar una diana efectiva para terapias antitumorales.

INTRODUCTION

1. BREAST CANCER

Breast cancer is the tumor with the highest prevalence among women, and the second in frequency worldwide after lung cancer. Breast cancer incidence increases at a rate of 1-2% per year, due to improved diagnostic methods and population aging. It is estimated that 1 of every 8 women will suffer from breast cancer in the coming years.

1.1. The normal mammary gland

The normal human mammary gland consists of a branching ductal-lobular system that organizes into acini. The mammary acinus consists of a central layer of luminal epithelial cells surrounded by a layer of myoepithelial cells and the basement membrane, a specialized laminin-enriched form of extracellular matrix (ECM, (Nelson and Bissell, 2006; Weigelt and Bissell, 2008); **Figure 1**).

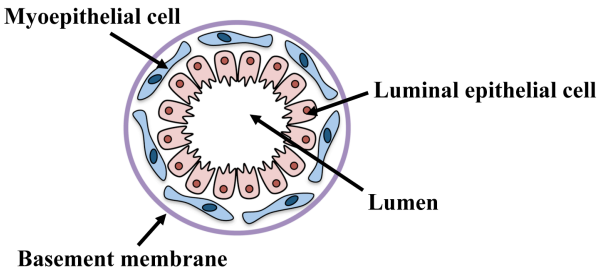


Figure 1. A mammary acinus. The mammary acinus consists of a layer of luminal epithelial cells that form the lumen, surrounded by basal myoepithelial cells. The acinus lies on a specialized form of ECM called the basement membrane.

Unlike other organs, most mammary gland development takes place postnatally, during puberty. In females, a surge of steroid hormones induces the mammary ductal rudiment present at birth to undergo a burst of branching morphogenesis (Nelson and Bissell, 2006). There is increasing evidence that, in analogy to the hematopoietic compartment, the adult mammary gland has a differentiation hierarchy. A better characterization of the distinct populations would help to identify the so-called cancer-initiating cells and would contribute to the design of more effective therapies (Stingl and Caldas, 2007; Visvader, 2009).

1.2. Breast cancer: classification and clinical implications

Breast cancer is a complex and heterogeneous disease, traditionally classified based on clinical and pathological features to predict outcome and treatment response. Three types of breast tumors can be distinguished immunohistochemically, according to their

expression of hormone receptors (HR; estrogen receptor/ER, progesterone receptor/PR) and/or the overexpression of the receptor tyrosine kinase (RTK) ERBB2/HER2.

Although valid, this classification has proved insufficient for predicting clinical outcome. Based on gene expression analysis, recent studies propose a further subdivision of some of these groups, leading to a new classification of breast tumors that includes six subtypes (Eroles et al., 2011) (**Table 1**).

Tumor cluster	Frequency	Characteristics		Prognosis	Treatment	Clinical equivalent
Luminal A	50-60%	Low mitogenic	Luminal cell gene expression pattern Cytokeratins 8/18	Good	Anti-estrogen therapy	HR+ (ER+/PR+)
Luminal B	10-20%	Highly mitogenic		Intermediate		
HER2	10-15%	Amplification of *ERBB2* amplicon (17q22.24) HER2 receptor overexpression Low ER expression		Poor	Anti-HER2 antibodies	HER2+
Basal	10-20%	Lack of HR expression or associated genes HER2-negative High expression of cytokeratins 5/6, 17, laminin and fatty acid-binding protein 7		Poor	Chemotherapy (No targeted treatment available)	TN (Not 100% concordant)
Normal-like	5-10%	Basal and adipose cell gene expression pattern		Intermediate		
Claudin-low	12-14%	Low expression of cell-cell contact genes Mesenchymal gene overexpression High immune cell infiltration		Poor		

Table 1. Breast cancer subtypes according to gene expression analysis. Gene expression analysis distinguishes six types of breast tumors that are indicators of disease prognosis. The table summarizes specific therapies and correlation with the traditional classification. Modified from (Eroles et al., 2011).

Improvement in diagnostic and classification procedures of breast cancer, as well as the development of oriented therapies, has contributed to increasing the survival rate of patients suffering from this disease. Resistance to current therapies is an important setback for clinicians and patients alike; this resistance can be constitutive (genetic), or acquired as a result of treatment. A better understanding of the molecular mechanisms responsible for therapy resistance, as well as the identification of specific targets to treat triple-negative (TN) breast cancer, represent an important research area in the field.

2. LIPID METABOLISM IN TUMORS

Oncogenic alterations lead to chronic, often uncontrolled cell proliferation. Emerging evidence suggests that metabolic reprogramming is a prerequisite for the rapid proliferation of cancer cells. This normally involves the consumption of additional nutrients and their diversion into macromolecular synthesis pathways.

2.1. Readjustment of lipid metabolism in tumors

The first observation regarding metabolic readjustment was that of Warburg in the 1930s, who described that tumor cells consume glucose through glycolysis (the conversion of glucose to pyruvate) at an abnormally high rate, and secrete most of the glucose-derived carbon as lactate rather than oxidizing it in the mitochondria in the tricarboxylic acid (TCA) cycle. Further studies established that the glycolytic use of glucose provides cancer cells with intermediates necessary for biosynthetic pathways that include lipid biosynthesis, needed for continuous generation of membranes in rapidly proliferating cells. In addition, glycolysis can bifurcate into the pentose phosphate pathway. This metabolic shunt generates ribose sugars, required for nucleotide synthesis, but also produces NADPH, which is needed for lipid biosynthesis and also acts as a reducing agent (DeBerardinis et al., 2008).

2.2. Lipid biosynthesis

Untransformed quiescent cells supply their fatty acid (FA) and cholesterol requirements from the extracellular environment. In tumor cells, their uncontrolled proliferation forces them to obtain most lipids through *de novo* synthesis. During proliferation, part of the glycolysis-derived pyruvate is converted to acetyl-CoA that enters the TCA cycle, where it is converted into intermediates such as citrate, used in lipid biosynthesis. After importation to the cytosol, citrate is cleaved by the enzyme ATP citrate lyase; the resulting acetyl-CoA is used for synthesis of FA and cholesterol (**Figure 2**).

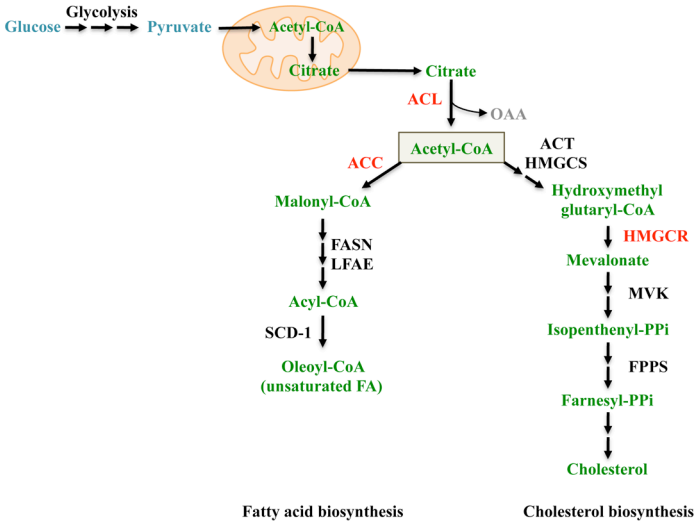


Figure 2. Lipid biosynthesis pathways. Fatty acid and cholesterol are both synthesized from acetyl-CoA. Acetyl-CoA is generated after cleavage of citrate, a metabolite generated in the mitochondria after glucose consumption via glycolysis; the diagram shows the enzymes responsible for the distinct steps. Enzymes highlighted in red are those whose control regulates pathway flow.

The activity of lipogenic enzymes is precisely regulated by cell metabolites through allosteric mechanisms, posttranslational modifications, and changes in their transcription levels. This guarantees the correct pathway flow and its appropriate regulation. Several of the enzymes implicated in this pathway are overexpressed in tumors, and their activity is often increased as a result of oncogenic transformation, contributing to the so called "lipogenic phenotype" of cancer (Menendez and Lupu, 2007).

2.3. Transcriptional regulation of lipid biosynthesis

Several enzymes involved in lipid biosynthesis are transcriptionally regulated, as stated above. This regulation is achieved through the sterol-regulatory element binding proteins (SREBP), which belong to the basic helix-loop-helix-leucine zipper family of transcription factors. The SREBP are synthesized as immature precursors in the endoplasmic reticulum and transported to the Golgi apparatus, where mature, active forms are generated by the sequential action of two proteases. The mature proteins enter the nucleus and promote expression of several targets, including SREBP itself (**Table 2**). Mammals express two SREBP isoforms, SREBP-1 and -2, which regulate FA/phospholipid and cholesterol biosynthesis, respectively. Both SREBP are activated downstream of the Akt/mammalian target of rapamycin (mTOR) pathway, providing a

direct link between this pathway, frequently mutated in cancer, and the lipogenic metabolism of tumors (Krycer et al., 2010).

SREBP-1	SREBP-2
SREBP-1	SREBP-2
ATP citrate lyase (ACL)	ATP citrate lyase (ACL)
Acetyl-CoA carboxylase (ACC)	Acetocetyl-CoA thiolase (ACT)
Fatty acid synthase (FASN)	Hydroxymethylglutaryl-CoA synthase (HMGCS)
Long chain fatty acid elongase (LFAE)	Hydroxymethylglutaryl-CoA reductase (HMGCR)
Stearoyl-CoA desaturase (SCD)	Mevalonate kinase (MVK)
	Farnesyl pyrophosphate synthase (FPPS)

Table 2. SREBP targets. SREBP transcription factors include two isoforms, SREBP-1 and -2. SREBP control the transcription of genes with sterol regulatory elements in the promoter; these include genes that encode several enzymes involved in fatty acid (SREBP-1 targets) and cholesterol biosynthesis (SREBP-2 targets).

The SREBP pathway is highly conserved in metazoans, where it coordinates lipid metabolism with other cell functions, allowing rapid adaptation for survival and growth (Osborne and Espenshade, 2009). One feature conserved in SREBP activation is its regulation by lipids, which guarantees homeostatic control of lipid synthesis. SREBP-2 processing is activated only when cholesterol levels at the endoplasmic reticulum drop below a threshold, whereas SREBP-1 responds to changes in oxysterol levels. Recent studies link SREBP-1 activation to phosphatidylcholine (PtdCho) metabolism, such that reduction of PtdCho levels and/or the enzymes involved in its synthesis correlate with increased levels of active SREBP-1 (Walker et al., 2011). Other phospholipids can also participate, as is the case of phosphatidylethanolamine (PtdEtn) in *Drosophilla* (Dobrosotskaya et al., 2002).

2.4. The role of DAG and PA as metabolic precursors

Diacylglycerol (DAG) and phosphatidic acid (PA) are two lipids with important roles as membrane constituents and as biosynthetic precursors of phospholipids. In addition, they are potent signaling molecules. DAG and PA thus allow integral control of membrane biogenesis and dynamics with nutrient-sensing and cell growth.

Biosynthetic PA pools are formed mainly by esterification at the C1 and C2 positions of glycerol-3-phosphate, a derivative of glycolysis intermediates, in a reaction that takes place at the endoplasmic reticulum. PA is an essential intermediate for the formation of other phospholipids and is a direct precursor of phosphatidylinositol (PtdIns) synthesis. This process involves PA conversion into the activated DAG form CDP-DAG, and the transfer of an inositol molecule (Loewen, 2012; Stace and Ktistakis, 2006). Finally, the sequential action of several PtdIns kinases leads to the generation of

PtdIns-4,5-bisphosphate ($PtdInsP_2$; **Figure 3**). PA stimulates the activity of the PtdIns-4-phosphate 5-kinase (PIP5K), which catalyzes the last step in $PtdInsP_2$ synthesis, an example of the coordination between the metabolic and effector functions of PA (van den Bout and Divecha, 2009).

PA can be dephosphorylated in the ER through the action of the PA phosphohydrolase (PAP), which leads to DAG production. DAG is the precursor of PtdCho and PtdEtn, through a reaction that involves choline or ethanolamine transfer from their CDP-bound active intermediates to DAG. In addition, PtdCho and PtdEtn precede the synthesis of phosphatidylserine (PtdSer). DAG can be further esterified in the C3 position of the glycerol molecule, leading to production of triglycerides (TAG; (Carrasco and Merida, 2007)), a major constituent of lipid droplet organelles (**Figure 3**).

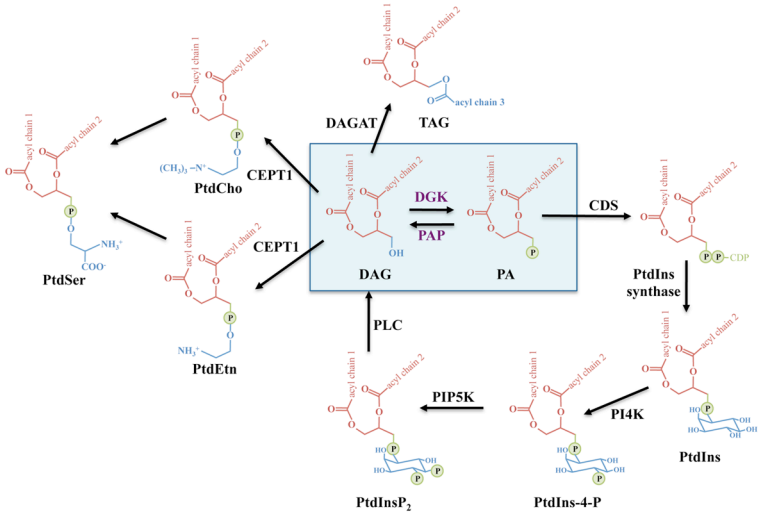


Figure 3. DAG and PA are central metabolites in phospholipid metabolism. DAG and PA are interconverted by DGK and PAP. PA is the precursor for PtdIns synthesis, which terminates in $PtdInsP_2$ generation. DAG is used to produce PtdCho, PtdEtn and PtdSer. Finally, DAG can be esterified into TAG, a form of metabolic energy storage.

In mammalian cells, additional DAG pools are generated in various compartments. In the Golgi apparatus, DAG is produced together with sphingomyelin through the activity of sphingomyelin synthase, which transforms ceramide and PtdCho in a reversible reaction. At the plasma membrane, receptor stimulation generates DAG, which behaves mainly as a second messenger through phospholipase C (PLC)-mediated hydrolysis of $PtdInsP_2$. Finally, DAG conversion into PA through diacylglycerol kinase (DGK) action is the first step in $PtdInsP_2$ replenishment, in the so-called "PtdIns cycle", a reaction that also terminates DAG signaling.

3. LIPID-REGULATED PATHWAYS IN CANCER

Protein binding to some lipids promotes conformational changes that trigger protein activation or inhibition in a membrane context. Signaling lipids control important cellular processes including proliferation, apoptosis, metabolism and migration. These lipids, as well as their modifying enzymes and downstream targets, are shared by many pathways and constitute a complex network. Imbalance of lipid-based signaling pathways contributes to progression of diseases such as cancer (Wymann and Schneiter, 2008).

3.1. The PI3K-Akt axis in breast cancer

The PtdIns-3-kinases (PI3K) are a family of enzymes that phosphorylate the 3'-position of the inositol ring of phosphatidylinositols. They are divided into three classes based on structural features and lipid substrate preference. To date, class I PI3K contribution to cancer is the best understood, and will be discussed here.

Class I PI3K are heterodimers composed of a catalytic and regulatory subunits that catalyze $PtdInsP_2$ conversion into PtdIns-3,4,5-trisphosphate ($PtdInsP_3$). This reaction is reversed by the action of the phosphatase and tensin homolog (PTEN), which specifically dephosphorylates $PtdInsP_3$ to again produce $PtdInsP_2$ (Bunney and Katan, 2010; Vanhaesebroeck et al., 2010).

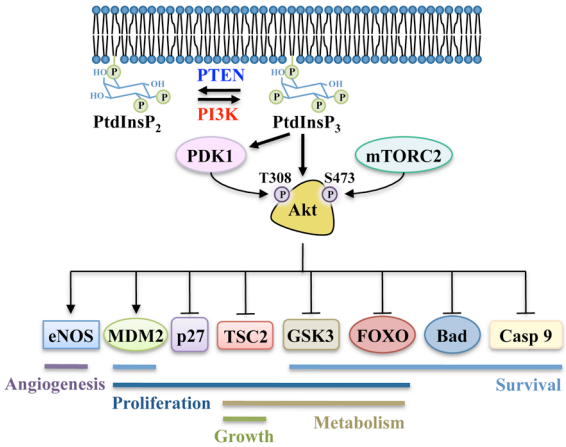


Figure 4. The PI3K/Akt axis. PI3K activation leads to $PtdInsP_2$ phosphorylation, which generates $PtdInsP_3$. This reaction is reversed by the PTEN phosphatase. $PtdInsP_3$ triggers PDK1 and Akt translocation to the membrane, where PDK1 phosphorylates and partially activates Akt. Akt phosphorylation by mTORC2 fully activates Akt protein kinase activity. Through phosphorylation of several effectors, Akt has a central role in the control of processes such as proliferation and metabolism.

$PtdInsP_3$ is almost undetectable in unstimulated cells, and is thus an optimal second messenger. RTK or G protein-coupled receptors trigger PI3K activation by releasing

the inhibitory input of the regulatory subunit over the catalytic domain. PI3K activity is further increased by the action of the Ras oncoproteins, leading to the generation and transient accumulation of $PtdInsP_3$ at the plasma membrane. This lipid is recognized by proteins that bear a specific lipid-binding module known as the pleckstrin homology (PH) domain, which makes them translocate to the plasma membrane (**Figure 4**).

The best-characterized PI3K pathway effector is the Ser/Thr kinase Akt (also referred to as PKB). To render Akt fully active, in addition to its $PtdInsP_3$-dependent translocation, this protein must be phosphorylated in two sites; the Thr308, mediated by phosphoinositide-dependent protein kinase 1 (PDK1), and by the mTOR complex 2 (mTORC2) in the Ser473. Active Akt in turn phosphorylates several proteins and regulates various biological processes such as cell survival, proliferation, metabolic control and angiogenesis (Manning and Cantley, 2007); **Figure 4**).

3.1.1. PI3K/Akt axis alterations in breast cancer

The PI3K axis is the second most altered pathway in cancer after that of p53. Several alterations lead to aberrant activation of this axis in breast cancer (**Table 3**).

Alteration	Frequency	Opportunity for therapeutic intervention
RTK		
ERBB2 amplification	18-25%	Tyrosine kinase inhibitors Monoclonal antibodies
PI3K		
PIK3CA (p110α) amplification	20-30%	Pan-PI3K catalytic inhibitors PI3K isoform-specific catalytic inhibitors Dual PI3K/mTOR inhibitors
PIK3CA (p110α) mutations	8.7%	
PIK3CB (p110β) amplification	5%	
PIK3R1 (p85α) mutations	Unknown	
PTEN		
PTEN protein loss	23-26%	No
PTEN mutations	6%	
PDK1		
PDPK1 amplification	20%	Activity inhibitors
Akt		
Akt1 E17K mutation	4-10%	Activity inhibitors
Akt2 amplification	3%	PH domain-directed inhibitors

Table 3. Alterations that lead to aberrant activation of the PI3K/Akt axis in breast cancer

Alterations in PI3K or PTEN tend to be mutually exclusive, and in breast cancer are normally associated with tumor type: luminal-like (ER^+PR^+) tumors normally bear mutations in or amplification of the PI3K genes, whereas alterations in PTEN are generally found in ER^-PR^- cancers (Saal et al., 2005). Both of these alterations can coexist with others, e.g., overexpression of the ERBB2 receptor, and provide constitutive resistance to some therapies.

3.2. The PA-mTOR axis: an explanation for rapamycin resistance

mTOR is a Ser/Thr kinase named after the finding that it is the target of the drug rapamycin, a macrolid with potent immunosuppressant activity that inhibits mTOR. mTOR integrates several intra- and extracellular inputs, such as mitogens, nutrient availability or oxygen status; to co-ordinately regulate cell growth and metabolism.

mTOR associates with various proteins to generate two structurally and functionally distinct complexes termed mTORC1 and mTORC2 (**Figure 5**). mTORC1 activates protein and lipid synthesis, nutrient transport and cell cycle progression. mTORC2 mediates cytoskeleton remodeling, transcription and metabolism control, and promotes cell survival. These two complexes differ in their response to rapamycin; mTORC1 is acutely sensitive to inhibition by rapamycin, whereas mTORC2 can show sensitivity after prolonged rapamycin treatment (Guertin and Sabatini, 2007; Laplante and Sabatini, 2009). Mitogenic stimulation of mTOR places this kinase in the PI3K/Akt axis, and situates mTOR complexes both down- and upstream of Akt. This pathway is regulated by negative feedback mechanisms that implicate mTORC1-dependent inhibition of PI3K and mTORC2 (**Figure 5**).

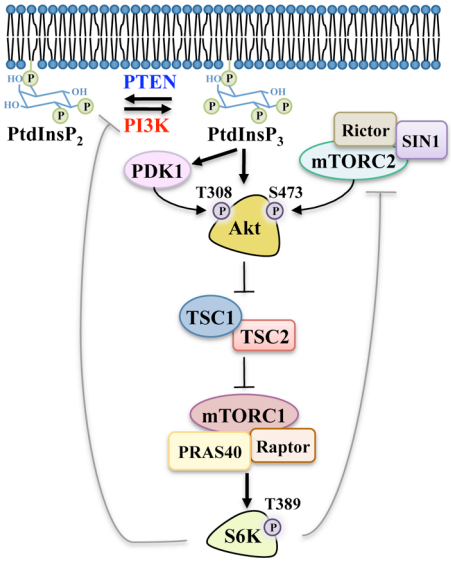


Figure 5. The PI3K/Akt/mTOR axis. mTOR nucleates two complexes of distinct protein composition. mTORC2 phosphorylates and promotes Akt activation. Akt phosphorylates and inhibits the tuberous sclerosis complex (TSC), an upstream inhibitor of mTORC1. mTORC1 triggers activation of S6K, a kinase involved in cell growth. S6K phosphorylates tyrosine kinase receptors and Rictor, leading to inhibition of PI3K and mTORC2, respectively. This feedback mechanism guarantees correct activation of the axis.

Mitogenic activation of mTOR requires PA. At difference from other lipids with second messenger functions, PA effectors in general do not appear to have a well-

defined lipid-recognition module, and use positively charged amino acids that interact with the negatively charged monoester headgroup of PA (Shin and Loewen, 2011; Stace and Ktistakis, 2006). mTOR binds PA through its FKBP-rapamycin binding (FRB) domain, an interaction that has been structurally characterized by nuclear magnetic resonance (NMR) (Veverka et al., 2008). PA increases mTOR catalytic activity after mitogenic stimulation, and stabilizes mTOR complexes (Fang et al., 2001; Toschi et al., 2009). The PA-producing enzymes phospholipase D (PLD) and DGK mediate mTOR activation (Avila-Flores et al., 2005; Chen et al., 2003; Sun et al., 2008).

As can be inferred from the above, rapamycin and PA share their mTOR binding region. Since mTOR is the key to control of cell functions, rapamycin semi-synthetic analogues (commonly referred as rapalogues, with improved pharmacokinetic properties) were candidate drugs for cancer treatment. Despite the initial promise of mTOR inhibition as a broad-spectrum anti-cancer strategy, the clinical performance of rapalogues has fallen short of expectations. Their effect is attributed to the disruption of mTORC1-derived negative feedback to PI3K, and to the fact that, in certain types of cells, rapamycin poorly inhibits mTORC2, which translates to increased Akt activation (Benjamin et al., 2011).

Tumors can also become refractory to rapalogue treatment by increasing their levels of PA, which then competes with the drugs for mTOR binding. In addition, the aggressive types of some tumors such as breast cancer have higher PA levels than their less-aggressive counterparts (Chen et al., 2005), and would require higher rapalogue doses to inhibit mTOR. Focus on PA-producing enzymes could thus be an effective new approach to mTOR pathway targeting in cancer.

3.3. PA-mediated regulation of the Ras-ERK axis

The extracellular signal-regulated kinase (ERK, also termed mitogen-activated protein kinase/MAPK) is a major effector of the Ras oncoprotein. This pathway consists of a GTPase-regulated kinase (MAPKKK) that phosphorylates an intermediate kinase (MAPKK) that, in turn, phosphorylates and activates an effector kinase (MAPK). In the ERK-MAPK pathway, these components are Ras, Raf, MEK and ERK, respectively. Hyperactivation of this axis has been causally linked to acquisition of the transformed phenotype.

The concept that PA regulates the ERK cascade was first proposed by Ghosh and coworkers in 1996 (Ghosh et al., 1996). These authors demonstrated that PA promoted

Raf-1 translocation to the plasma membrane, where it interacts with Ras and is activated. PA nonetheless did not stimulate Raf kinase activity (Rizzo et al., 1999; Rizzo et al., 2000). Zhao et al. showed that Ras activation by the guanine exchange factor (GEF) Son of sevenless (Sos) is PA-dependent. They demonstrated that Sos interacts with PA with high affinity and specificity via its PH domain, and that this interaction is essential for epidermal growth factor (EGF)-induced Sos recruitment and Ras activation (Zhao et al., 2007).

Finally, PA was recently shown to positively modulate the RAB-coupling protein (RCP; (Rainero et al., 2012)), which induces motility, invasion and increased survival of breast cancer cells; its effects can be mediated by increasing H-Ras and MAPK activities (Zhang et al., 2009). RCP is also implicated in the traffic of adhesion receptors, and contributes to integrin-mediated MAPK and PI3K activation in a β-integrin chain-specific manner (Mills et al., 2009).

3.4. The PKC-PKD axis in breast cancer

Protein kinase C (PKC) and protein kinase D (PKD) are important DAG sensors. DAG signals are sensed by proteins that contain C1 domains, which are zinc-finger domains of approximately 50 amino acids, enriched in cysteine and histidine residues. A battery of proteins, including kinases and non-kinases, respond to DAG generation (Carrasco and Merida, 2007).

The PKC are a Ser/Thr kinase family that comprises 2% of the human kinome. They are encoded by nine genes, and are subdivided into three groups according to their allosteric activation mechanism. Both classical (cPKC) and novel PKC (nPKC) respond to DAG generation, and cPKC to Ca^{2+} binding as well. Atypical PKC (aPKC) activation is DAG-independent (Rosse et al., 2010).

The PKC family regulates a wide variety of biological processes, including cell proliferation, survival and migration. Given the large number of isoforms, however, it has not been possible to determine function common to all of them. In addition, PKC isozymes have distinct roles depending on cell type (Griner and Kazanietz, 2007). Mutations in PKC isozymes are rare in the context of cancer. PKC deregulation has been observed in breast cancer, and several isoforms contribute to its progression, with roles as tumor promoters or suppressors, depending on tumor context (**Table 4**; (Urtreger et al., 2012).

Isoform	Expression	Role	Mechanism
PKCα	Increased/ decreased	Promoter	Induces multi-drug resistance (ER^+ tumors) Promotes proliferation and migration
		Suppressor	Reduces proliferation
PKCβ	Unknown	Promoter	Promotes cell growth and angiogenesis
		Suppressor	Reduces protease expression
PKCδ	Unknown	Promoter	Promotes proliferation and metastasis; induces anti-estrogen resistance
		Suppressor	Increases apoptosis
PKCε	Increased	Promoter	Promotes tumor growth, metastasis
PKCη	Increased (in ER^- breast tumors)	Promoter	Increases cell survival; correlates with lymph node status
PKCθ	Unknown	Promoter	Promotes cell growth, survival and invasion
PKCζ	Unknown	Promoter	Increases proliferative and invasive potential
PKCι	Increased	Promoter	Suppresses senescence

Table 4. PKC isoforms involved in breast cancer. Summary of PKC isoforms linked to breast cancer progression and the mechanisms implicated in their role as tumor suppressors or promoters. Modified from (Urtreger et al., 2012)

The best-characterized PKC isoforms in breast cancer are cPKCα and nPKCε. PKCα levels correlate with ER negativity, and high levels of this isoform predict a poor response to anti-estrogen therapy (Assender et al., 2007). PKCα promotes tumor proliferation and migration, although the exact mechanism by which it contributes to breast cancer progression remains to be determined (Lonne et al., 2010). PKCε belongs to the nPKC group, and is thought to be regulated both by DAG and PA (Corbalan-Garcia et al., 2003). PKCε levels are normally increased in breast tumor samples and its expression correlates with tumor aggressiveness, as it promotes proliferation as well as invasion (Pan et al., 2005).

PKD was initially classified as a PKC (PKCμ) because of its DAG dependence, but is now considered a separate family. PKD are both DAG and PKC effectors that control cell survival and motility, as well as Golgi vesicle fission and transport, among other processes (Fu and Rubin, 2011). To date, three PKD isoforms have been identified in human, but only PKD1 is well characterized in breast cancer. PKD1 levels are notably reduced in breast cancer-derived cells as they become more aggressive, as confirmed in patient samples; PKD1 also reduces matrix metalloprotease expression, and therefore inhibits breast cancer cell invasion (Eiseler et al., 2009). This role is at

variance with that described in pancreatic or cervical cancers, for example, in which PKD1 has a tumor-promoting function.

3.5. DAG/PA offers new opportunities for breast cancer treatment

The previous summary shows how DAG and PA lie directly or indirectly at the core of several pathways whose aberrant activation causes or contributes to maintenance of the transformed phenotype. The excessive $PtdInsP_2$ consumption due to oncogenic PI3K hyperactivation or to lack of PTEN phosphatase, for instance, places an important demand on the cell for continuous PtdIns turnover, a PA-dependent process. Aberrant mTOR activation might also need an enhanced PA supply, and several lines of evidence point to a central role for PA in Ras activation. In addition, the lipogenic metabolism of tumors requires continuous phospholipid biosynthesis to generate membranes. The DGK balance DAG and PA levels, and have been implicated in the pathogenesis of several diseases. A closer examination of DGK-mediated functions in cancer would help to address the potential of this pathway as a pharmacological target.

4. DIACYLGLYCEROL KINASES

The DGK are a family of lipid enzymes that phosphorylate DAG to generate PA. DGK activity was first described in rat brain microsomes in the classical studies by Hokin and Hokin (1959) (Hokin and Hokin, 1959), which led to characterization of the PtdIns cycle. Following those early studies, the DGK were shown to be a large family of lipid kinases, present from bacteria to mammals, that are central to the regulation of cell responses; they thus offer opportunities for therapeutic intervention in human disorders (**Figure 6**).

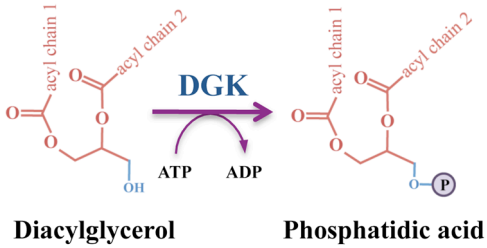


Figure 6. The DGK reaction. The DGK are enzymes that catalyze transfer of the ATP γ-phosphate group to DAG, generating PA.

4.1. Classification

The DGK species were initially classified according to their enzymological properties, based on sensitivity to several activator or inhibitory compounds (Kanoh et al., 1990). To date, the ten known members of the mammalian DGK family are classified into five subtypes, based on the distinct regulatory domains in their primary sequence (**Figure 7**); this diversity is further increased by alternative splicing of some isoforms (Merida et al., 2008).

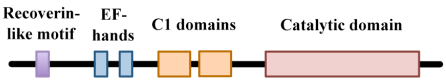
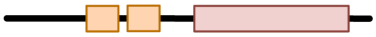
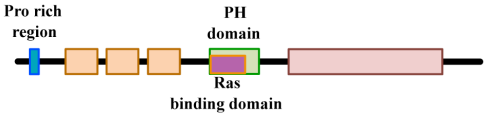
Type	Isoforms	Structural domains	Regulation
I	DGKα DGKβ DGKγ		Ca^{2+}, PtdSer, $PtdInsP_3$, Src, Lck, PKC
II	DGKδ DGKη DGKκ		PKC, phospholipids, oligomerization
III	DGKε		Phospholipids
IV	DGKζ DGKι		PKC, Src, Rb, leptin, protein interaction
V	DGKθ		PtdSer, PA, RhoA

Figure 7. The DGK family. The DGK family is formed of ten isoforms, classified into five subtypes based on domain structure. This generates a great diversity of DGK that are regulated by diverse stimuli. Modified from (Merida et al., 2008).

4.2. Structure

All mammalian DGK share two structural features, a catalytic domain and at least two C1 domains; the former is divided into conserved and accessory subdomains. The conserved catalytic domain resembles those of ceramide and sphingosine kinases, whereas the accessory subdomain is unique to DGK. In most cases, these subdomains constitute an uninterrupted catalytic domain, except for type II DGK. The catalytic domain has a GGDGXXG motif, similar to the ATP-binding site of protein kinases (Merida et al., 2008).

The DGK have two C1 domains, except for type V DGKθ, which has three. Sequence analysis indicated that with the exception of the first C1 domain of DGKβ and DGKγ, the DGK C1 region lacks the key residues that define a canonical C1-like phorbol ester-binding domain. These regions nonetheless contribute to DGK function, as mutations within these domains reduce enzyme kinase activity. Rather than binding

DAG, it is likely that the DGK C1 domains act as protein-protein interaction sites; for example, they bind to the GPCR adaptors β-arrestins (Nelson et al., 2007). The DGK have other non-conserved structural domains, distinct for each of the five subtypes, that appear to have regulatory functions (Merida et al., 2008) (Cai et al., 2009) (van Blitterswijk and Houssa, 2000) (**Figure 7**).

4.3. Diacylglycerol kinases functions

In early studies, the main function attributed to DGK was that of negative regulation of PKC signaling. With the characterization of new DAG receptors and the discovery of PA-regulated proteins, our understanding of DGK function has broadened. In addition, some DGK functions are independent of their lipid kinase activity, and are probably depend on their role as scaffolds.

DGK regulation of both DAG and PA levels, together with their tissue-specific expression pattern and their regulation by a variety of stimuli, makes the study of DGK functions extremely complex. A single DGK isoform might have opposite roles in different systems, and/or distinct DGK can have redundant or opposite roles in the same system. Specific DGK isoforms are implicated in many biological processes, and mutation or the lack of some isoforms are linked to human diseases (**Table 5**).

Subtype	Isoform	Tissue distribution	Function (inferred from knockout mice)
I	**DGKα**	T cells, brain (oligodendrocytes), kidney, lung, testis	Negative regulator of TCR signaling Anergy induction
	DGKβ	Brain (neurons), cardiac muscle	Mood disorders
II	**DGKδ**	Ubiquitous (except brain)	Insulin resistance
	DGKη	Ubiquitous	Bipolar disorder
III	**DGKε**	Ubiquitous	PtdIns turnover
IV	**DGKζ**	Ubiquitous	Cardiac hypertrophy Ischemic response Dendritic spine maintenance Negative regulator of TCR signaling
	DGKι	Brain, retina, placenta, ovary	Neurotransmitter release Ras promotion

Table 5. Functions attributed to the DGK family. Most DGK proteins have a tissue-specific expression pattern. Characterization of mice lacking certain isoforms implicates some members of this lipid kinase family in the origin of some diseases.

4.4. Diacylglycerol kinases and cancer

The earliest reports suggesting deregulated DGK activity in transformed cells came from studies in the 1980s that showed increased DGK activity in Src- and Erbb-transformed cells (Kato et al., 1987; Sugimoto et al., 1984), although this was not followed by further studies on the DGK contribution to the transformed phenotype.

The first DGK implicated in cancer was the type IV DGKι. Mice deficient in this isoform developed fewer tumors when on a Ras-dependent background (Regier et al., 2005). Despite this initial description, the best-characterized DGK in cancer is the type I DGKα. DGKα is an important effector of hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) receptors (Cutrupi et al., 2000) (Baldanzi et al., 2004); it is also essential for survival in lymphomas bearing the anaplastic lymphoma kinase (ALK; (Bacchiocchi et al., 2005)). In addition, DGKα contributes to the progression of endometrial and hepatic cancer (Filigheddu et al., 2011) (Takeishi et al., 2012).

4.5. DGKα

DGKα was the first mammalian DGK to be isolated and characterized. It is a type I DGK, and thus has N-terminal Ca^{2+}-binding EF hand domains and a recoverin-like motif, both important in its regulation.

4.5.1 Transcriptional regulation of the *DGKα* gene

In addition to oligodendrocyte specificity, DGKα expression is particularly enriched in the thymus and peripheral T cells. DGKα is also expressed strongly in spleen, lung and testis, whereas its levels are low in liver, kidney and heart. Initial characterization of the *DGKα* gene provided early insight into the regulatory regions that control this tissue-specific expression (Fujikawa et al., 1993).

Several cDNA microarray analyses also indicate that diverse signaling pathways control *DGKα* gene transcription. p53 and PPARγ promote DGKα mRNA upregulation after treatment with DNA-damaging agents or anti-inflammatory drugs, respectively (Kannan et al., 2001; Verrier et al., 2004). High DGKα levels appear to correlate with differentiated or quiescent states (Merida et al., 2009), whereas reduced DGKα mRNA levels are described after cell treatment with proliferative stimuli, such as IL-2 in $CD8^+$ T cells or FBS in fibroblasts (Gu and Iyer, 2006; Verdeil et al., 2006).

Some studies have assessed DGKα expression in cancer. This isoform is upregulated in primary gastric adenocarcinomas and in breast cancer metastases (Hao et al., 2004) (Marchet et al., 2007). An interesting observation was made by (Berrar et al., 2005), who identified DGKα expression as a good predictor of disease outcome in lung adenocarcinoma. In addition, *DGKα* is synergistic in mutated p53- and/or Ras-induced colon cancer (McMurray et al., 2008), and was identified as a causative gene in pancreatic cancer, in which *DGKα* mutations have been identified (Carter et al., 2010).

4.5.2. Regulation of DGKα activity

Studies of DGKα activation demonstrated that its membrane translocation is rapid and transient, a process that is more sustained for a kinase-defective version of the enzyme (Sanjuan et al., 2001). The N-terminal domain has a negative regulatory role, since its deletion led to constitutive DGKα localization at membranes. An increase in Ca^{2+} levels is nonetheless insufficient to promote DGKα membrane translocation in physiological conditions; DGKα must be phosphorylated on tyrosine to be fully active (Cipres et al., 2003; Sanjuan et al., 2001). Tyrosine 335, a residue that lies at the hinge between the C1 domain and the catalytic region, was identified as a major DGKα phosphorylation site and proved critical for its translocation to the membrane. Phosphorylation of this residue is mediated by tyrosine kinases of the Src non-receptor tyrosine kinase family (SFK; (Cutrupi et al., 2000), which phosphorylate DGKα in response to growth factors such as HGF (Baldanzi et al., 2008; Baldanzi et al., 2004). In T cells, the tyrosine kinase Lck, which is activated downstream of the T cell receptor (TCR), phosphorylates DGKα at Tyr335 (Merino et al., 2008). These data led to the proposal of an activation model for DGKα, in which Ca^{2+} elevation induces a conformational change that releases the negative regulation of the DGKα N-terminal domain. Tyrosine phosphorylation induces an active, open conformation that stimulates DGKα translocation to the membrane, where it phosphorylates DAG. In addition to dephosphorylation, PA production restores the closed, inactive conformation of DGKα and promotes its return to the cytosol.

4.5.3. DGKα functions

The functions initially attributed to DGKα were negative regulation of Ras activation in T cells through modulation of membrane binding, and activation of the Ras GEF Ras guanyl-releasing protein 1 (RasGRP1; (Sanjuan et al., 2003)). This idea was further reinforced by the hyperactive phenotype of T cells from DGKα-deficient mice

(Olenchock et al., 2006). Whereas DGKα functions in the immune system are related mainly to its role as a negative regulator of DAG signaling, several studies suggest that DGKα-produced PA is important for the survival, migration and invasive properties of tumors (**Table 6**).

Stimulus	Mediators	Effect	References
HGF	Rac aPKC RhoGDI	Migration	(Chianale et al., 2010)
VEGF	-	Angiogenesis Cell proliferation	(Baldanzi et al., 2004)
NPM-ALK	-	Anaplastic large-cell lymphoma survival	(Bacchiocchi et al., 2005)
TNFα	PKCζ/NFκB	Melanoma survival	(Kai et al., 2009; Yanagisawa et al., 2007)
17-β-estradiol	GRP30	Endometrial cancer proliferation, motility, survival	(Filigheddu et al., 2011)
Integrin	RCP	Invasive migration	(Rainero et al., 2012)

Table 6. Downstream effectors of DGKα-generated PA. Through its function as a PA producer, DGKα controls several pathways, most of which promote cell proliferation, survival and motility, and contribute to cancer progression.

4.6. DGKζ

DGKζ is one of the two type IV DGK, and is thus characterized by a MARCKS motif that overlaps with a nuclear localization sequence (NLS). DGKζ also has four C-terminal ankyrin repeats and a PDZ-binding domain, which allow the enzyme to interact with several proteins (Topham et al., 1998) (Rincon et al., 2007).

4.6.1. DGKζ expression

DGKζ was initially cloned from endothelial cells, rat brain and retina. Although it is a ubiquitous enzyme, its mRNA is expressed strongly in thymus, brain and skeletal muscle. Two alternatively spliced isoforms have been identified, which originate distinct proteins of 115 and 130 kDa; the latter has only been identified in skeletal muscle (Rincon et al., 2012).

4.6.2. Regulation of DGKζ activity and subcellular localization

DGKζ has a MARCKS motif, as mentioned above, that includes consensus PKC phosphorylation sites. Its phosphorylation by PKC, as well as its ability to form various complexes, modulates both DGKζ localization and activity (Cai et al., 2009) (Rincon et al., 2012).

Early studies in adherent cells identified DGKζ as a nuclear enzyme whose localization correlates with cell cycle phase. PKC-mediated phosphorylation of the DGKζ NLS was proposed to negatively regulate its nuclear localization (Topham et al., 1998). Nuclear DGKζ function has not been fully elucidated; it is thought to bind the retinoblastoma (Rb) protein, which increases its activity and downregulates cyclin D3 levels (Evangelisti et al., 2009; Los et al., 2007). In T cells, PKC-dependent phosphorylation of the MARCKS domain is necessary for DGKζ membrane translocation (Santos et al., 2002). In its C-terminal half, DGKζ also has an ERK phosphorylation consensus sequence (Avila-Flores et al., 2005), whose function remains unknown.

4.6.3. DGKζ functions

The extreme versatility of DGKζ is underlined by recent studies. This isoform clearly has a dual role, as it inhibits DAG-regulated enzymes and activates PA-mediated functions (Rincon et al., 2012). DGKζ acts a negative regulator of PKCα signal duration; the two proteins interact in a complex such that PKCα maintains DGKζ in a non-phosphorylated active form, allowing mutual regulation (Luo et al., 2003a, b). DGKζ-produced PA modulates several signaling effectors, one of which is PIP5K, an enzyme that catalyzes the final step of $PtdInsP_2$ synthesis (Luo et al., 2004). DGKζ also regulates Rac1, a Rho GTPase family member that regulates changes in the actin cytoskeleton via activation of p21-activated kinase 1 (PAK1; (Abramovici et al., 2009)). Another DGKζ effector is mTOR, a master integrator of signals and controller of cell growth (Avila-Flores et al., 2005).

DGKζ is strongly expressed in thymus and mature T cells, where it appears to have redundant functions with DGKα as a negative regulator of the DAG/RasGRP1/Ras axis. In DGKζ-deficient mice, the Ras/ERK pathway is hyperactive (Zhong et al., 2003). Despite this apparent redundancy, the contribution of both isoforms to DAG-regulated functions in T lymphocytes seems to differ; for instance, recent experiments showed that DGKζ is the main isoform responsible for DAG metabolism at the immune synapse (Gharbi et al., 2011).

Through several mechanisms, DGKζ can thus participate in various biological processes, including cardiac remodeling, neuron function, T cell response and innate immunity (Rincon et al., 2012)

OBJECTIVES

Diacylglycerol kinases (DGK) balance the levels of diacylglycerol and phosphatidic acid (PA), two lipids that lie at the core of several metabolic and signaling pathways involved in breast cancer progression. While various studies implicate DGKα-generated PA in several cancer processes, there are no data for DGKζ. The redundancy between these two isoforms makes it necessary to address the possible DGKζ contribution to the maintenance of breast cancer. Our overall objective is to **characterize the implication of the DGK pathway in breast cancer physiopathology**.

To achieve this aim, we propose the following specific objectives:

1. Determine and compare DGKα and DGKζ expression and activity in breast cancer and the normal mammary gland, and correlate them with those of their regulators.

2. Assess the contribution of DGKα and DGKζ to breast cancer progression.
   - 2.1 Evaluate DGK-mediated control of the SREBP (sterol regulatory element-binding proteins) pathway in breast cancer.
   - 2.2 Examine the contribution of the DGK pathway to the maintenance of the oncogenic axes that promote breast cancer progression (PI3K/Akt/mTOR, Ras/ERK, ERBB2/Src).
   - 2.3 Examine the contribution of the DAG/PA axis to the development of pharmacological resistance in breast cancer cells.

3. Study the relevance of DGK expression and activity for breast cancer initiation and progression *in vivo*.

4. Establish a reference framework to evaluate the potential of the DGK pathway as a target for therapeutic intervention.

MATERIAL AND METHODS

1. Cell lines

The MCF-10A, MCF-7 and MDA-MB-231 cell lines were purchased from the ATCC. The cell lines MDA-MB-468, T-47D and Hs 578T were kindly donated by Dr. Lourdes Planelles (CNB/CSIC, Madrid), BT-474 by Dr. Santos Mañes (CNB/CSIC, Madrid), SK-BR-3 by Dr. Javier Benítez (CNIO, Madrid). The normal luminal cell line HB4a was kindly donated by Dr. Hugh T. Reyburn (CNB/CSIC). A summary of the characteristics of the breast-derived cell lines as well as their culture conditions are detailed in **Table 7**. All cell lines were maintained at 37ºC and 5% CO_2, except those cultured in Leibovitz's L15, where CO_2 was absent.

2. Antibodies

Antibody	Supplier	Antibody	Supplier
Goat anti-mouse HRP	Dako	Anti-pAkt Ser473	Cell Signaling
Goat anti-rabbit HRP	Dako	Anti-pAktThr308	Cell Signaling
Anti-DGKα	Abnova	Anti-Akt	Cell Signaling
Anti-DGKζ	Donated by MK Topham	Anti-mTOR	Cell Signaling
Anti-tubulin	Sigma	Anti-Rictor	Bethyl Laboratories
Anti-FoxO3a	Cell Signaling	Anti-Raptor	Cell Signaling
Anti-FoxO1	Cell Signaling	Anti-GAPDH	Santa Cruz Biotechnology
Anti-pFOXO1/3a Thr24/Thr32	Cell Signaling	Anti-PARP	Cell Signaling
Anti-β-actin	Sigma	Anti-pSrc Tyr416	Cell Signaling
Anti-HER2	Cell Signaling	Anti-Src	Cell Signaling
Anti-phospho Tyr (4G10)	Upstate	Anti-pERK Tyr202/Thr204	Cell Signaling
Anti-pSer PKC substrates	Cell Signaling	Anti-ERK	Cell Signaling
Anti-pPanPKC (γThr514)	Cell Signaling	Anti-HIF1α	BD Biosciences
Anti-pPanPKC (βSer660)	Cell Signaling	Anti-ILK	BD Biosciences
Anti-PKCα (C-20)	Santa Cruz Biotechnology	Anti-GM130	BD Biosciences
Anti-pPKD Ser744/8	Cell Signaling	Anti-CD49f-AF488	Beckman Coulter
Anti-PKD	Santa Cruz Biotechnology	Anti-CD49b-PE	Beckman Coulter
Anti-SREBP1	Millipore	Anti-Sca1-PECy7	Beckman Coulter
Anti-pP70S6K Thr389	Cell Signaling	Anti-CD45-biotin	Beckman Coulter
Anti-P70S6K	Cell Signaling	Anti-CD31-biotin	Beckman Coulter
Anti-rpS6 Ser240/4	Cell Signaling	Anti-Ter119-biotin	Beckman Coulter
Anti-rpS6	Cell Signaling	Anti-BP1-biotin	Beckman Coulter
Anti-cyclin D3	Cell Signaling	Streptavidin APC-Cy7	Beckman Coulter

Cell line	Tissue type	Origin	Receptors (ER, PR, HER2)	Clinical subtype	Gene cluster	*TP53* status	PI3K pathway mutations	Culture medium
MCF-10A	Fibrocystic disease	Primary breast	Triple negative	TN	Basal B	WT	None	DMEN/F12 (1:1), 5% horse serum, 2 mM L-Gln, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 20 ng/mL EGF
HB4a	-	-	ER+/PR+	HR+	Luminal	N.D.	None	DMEM, 10% FBS, 2 mM L-Gln
MCF-7	IDC	Pleural effusion	ER+/PR+	HR+	Luminal	WT	p110α E545K	MEM, 10% FBS, 2 mM L-Gln, NEAA, sodium pyruvate
T-47D	IDC	Pleural effusion	ER+/PR+	HR+	Luminal	Mut	p110α H1047R	RPMI, 10% FBS, 2 mM L-Gln, NEAA, sodium pyruvate
BT-474	IDC	Primary breast	ER+/PR+ HER2 overexp.	HER2+	Luminal	WT	p110α K111N	RPMI, 10% FBS, 2 mM L-Gln, NEAA, sodium bicarbonate
SK-BR-3	AC	Pleural effusion	ER-/PR- HER2 overexp.	HER2+	Luminal	WT	None	DMEM, 10% FBS, 2 mM L-Gln
MDA-MB-231	AC	Pleural effusion	Triple negative	TN	Basal B	Mut	None	DMEM, 10% FBS, 2 mM L-Gln
MDA-MB-468	AC	Pleural effusion	Triple negative	TN	Basal A	WT	PTEN	Leibovitz's L-15, 10% FBS
Hs 578T	IDC	Primary breast	Triple negative	TN	Basal B	Mut	None	DMEM, 10% FBS, 2 mM L-Gln, 10 μg/mL Insulin

Table 7. Cell lines used in this study.

3. Reagents

Reagent	Supplier	Reagent	Supplier
Leupeptin	Roche	Dioctanoyl-DAG	Avanti Polar Lipids
Aprotinin	Roche	Dioleoyl-DAG	Avanti Polar Lipids
Triton X-100	Calbiochem	Dioctanoyl-PA	Avanti Polar Lipids
CHAPS	Calbiochem	Dioleoyl-PA	Avanti Polar Lipids
R59949	Calbiochem	Brain PtdSer	Avanti Polar Lipids
Bisindolylmaleimide	Calbiochem	Liver PtdIns	Avanti Polar Lipids
Gö6976	Calbiochem	Brain PtdIns-4-P	Avanti Polar Lipids
Rapamycin	LC Laboratories	[γ-^{32}P]ATP	Hartmann Analytic

Remaining reagents where purchased from Sigma, or as indicated. HPLC-grade solvents for lipid handling were acquired from Merck.

4. Western blot analysis

For western blot analysis, cells were lysed (4ºC, 15 min) in p70 buffer (10 mM Hepes pH 7.5, 15 mM KCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol and 0.2% NP-40) containing protease and phosphatase inhibitors (20 μM leupeptin, 1.5 μM aprotinin, 1 mM PMSF, 1 mM sodium orthovanadate, 40 mM β-glycerophosphate and 2 mM NaF). The lysate was then centrifuged (12000 xg, 15 min, 4ºC) and the supernatant was quantified with the Pierce 660 nm Protein Assay (Thermo Scientific). Samples were denatured in Laemmli buffer (Laemmli, 1970) and resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad) and incubated with appropriate antibodies.

5. Fractionation analysis

5.1. Membrane isolation

To purify cell membranes, cells were lysed in 50 mM Tris-HCl pH 7.5, with protease and phosphatase inhibitors. Lysates were centrifuged (800 xg, 15 min, 4ºC) to exclude nuclei and cell debris, and the remaining supernatant was further centrifuged (100000 xg, 1 h, 4ºC). The pellet, corresponding to the membrane fraction, was resuspended in 50 mM Tris-HCl pH 7.5 and used for activity assays.

5.2. Preparation of nuclear extracts

Nuclear extracts were obtained as described (Meyer et al., 2002). Cells were resuspended in TKM buffer (25 mM Tris-HCl pH 7.5, 5 mM KCl, 1 mM $MgCl_2$) and incubated (5 min, 4ºC) prior to addition of 1 vol TKM buffer containing 1% NP-40, followed by further incubation (5 min, 4ºC). Nuclei were sedimented (1000 xg, 5 min,

4ºC), and resuspended in TKM buffer containing 0,5% NP-40 and 100 mM NaCl. Nuclei were separated from inner membranes by centrifugation (2100 xg, 5 min) through a 3-volume cushion of 1.62 M sucrose in TKM buffer. The pelleted nuclei were washed with TKM buffer containing 0.5% NP-40 and 100 mM NaCl, and lysed in high salt RIPA buffer (20 mM Tris-HCl pH 7.5, 300 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate and 10% glycerol). All buffers contained protease and phosphatase inhibitors.

6. Immunoprecipitation

For protein-protein interaction analysis by immunoprecipitation, cells were lysed (30 min, 4ºC) in 50 mM Hepes pH 7.4, 150 mM NaCl, 1% NP-40 and 10% glycerol, containing protease and phosphatase inhibitors. Lysates (500 μg - 1 mg) were incubated (overnight, 4ºC) with the appropriate antibody, followed by incubation with 50 μl of 50% protein G-Sepharose slurry (1 h, 4ºC). Immunoprecipitates were washed thrice in lysis buffer and once with 0.5M LiCl. Immunoprecipitated proteins were resolved by SDS-PAGE and transferred to PVDF membranes for Western blot analysis.

When phosphotyrosine was immunoprecipitated, Triton X-100 was used instead of NP-40 and incubation time with antibody was reduced (3 h). Immunoprecipitated complexes were washed twice each with lysis buffer, washing buffer 1 (100 mM Hepes pH 7.0, 250 mM LiCl), washing buffer 2 (10 mM Hepes pH 7.0, 100 mM NaCl, 1 mM EDTA), and 10 mM Hepes pH 7.0. The resulting immunoprecipitates were used for Western blot analysis or activity assay, as described below.

6.1. mTOR immunoprecipitation

To detect mTOR complexes, immunoprecipitation was performed as described (Kim et al., 2002). Cells were lysed in 40 mM Hepes pH 7.5, 120 mM NaCl, 1 mM EDTA, 0.3% CHAPS, containing protease and phosphatase inhibitors. Lysates (500 μg) were incubated with anti-mTOR antibody (Cell Signaling, 1:100, overnight, 4ºC), followed by incubation with 50 μl of 50% protein G-Sepharose slurry (1 h, 4ºC). Immunoprecipitates were washed four times with lysis buffers and twice with 50 mM Hepes pH 7.5, 150 mM NaCl. Immunoprecipitated proteins were resolved by SDS-PAGE and transferred to PVDF membranes for Western blot analysis.

7. Plasmids, RNAi sequences and transfections

The following plasmids were used in the present study:

Plasmid	Reference/Supplier
pSUPER.retro-shRNA mouseDGKα (control)	A. Ávila-Flores
pSUPER.retro-shRNA humanDGKα	A. Ávila-Flores
pSUPER.retro-shRNA humanDGKζ	A. Ávila-Flores
pEFbosEGFP-C1bPKCθ	(Carrasco and Merida, 2004)
GFP-PH-CERT	Sima Lev; (Peretti et al., 2008)
pLKO-Tet-on-neo	(Wiederschain et al., 2009)/Addgene (#21916)
pRSV-Rev	John Stingl (CRI, Cambridge, UK)
pMDLg/pRRE	John Stingl (CRI, Cambridge, UK)
pVSVG	John Stingl (CRI, Cambridge, UK)

Plasmids were transfected with Lipofectamine LTX according to manufacturer's instructions. Assays were performed 48 h post-transfection. DGK-targeting RNAi oligonucleotides have been described (Avila-Flores et al., 2005) (Rincon et al., 2007) (Gharbi et al., 2011) and were transfected with Oligofectamine as recommended by the manufacturer. Effective DGK depletion was achieved at 72 h after transfection.

8. Establishment of Tet-on inducible cell lines MDA-MB-231-shRNA-control, -shRNA-DGKα, and -shRNA-DGKζ

DGK-targeting RNA sequences, detailed below, were cloned between the AgeI and EcoRI restriction sites of the pLKO-Tet-on-neo vector. Incorporation of the insert was verified after digestion with XhoI. Clones that contain the shRNA oligos yield two closely migrating bands at and below 200 bp. In addition, the presence of the insert was confirmed by sequencing using a primer (shRNASeq) that anneals upstream of the H1/TO promoter. Inducible insert expression was achieved by treating the cells with doxycyline (100 ng/mL, every 48 h). DGK reduction was effective after 96 h of doxycycline treatment. The oligonucleotides designed were as follows:

Oligonucleotide	Sequence
shRNA-mouse DGKAlpha sense	5' CCG GAC ACA AGA CCA CAG ATG ATA ACT CGA GTT ATC ATC TGT GGT CTT GTG TTT TTT 3'
shRNA-mouse DGKAlpha antisense	5' AAT TAA AAA ACA CAA GAC CAC AGA TGA TAA CTC GAG TTA TCA TCT GTG GTC TTG TGT 3'
shRNA-mouse DGKZeta sense	5' CCG GCT ATG TGA CGG AGA TTG CCA ACT CGA GTT GGC AAT CTC CGT CAC ATA GTT TTT 3'
shRNA-mouse DGKZeta antisense	5' AAT TAA AAA CTA TGT GAC GGA GAT TGC CAA CTC GAG TTG GCA ATC TCC GTC ACA TAG 3'

shRNA-human DGKAlpha sense	5' CCG GGC CAG AAG ACC ATG GAT GAA ACT CGA GTT TCA TCC ATG GTC TTC TGG CTT TTT 3'
shRNA-human DGKAlpha antisense	5' AAT TAA AAA GCC AGA AGA CCA TGG ATG AAA CTC GAG TTT CAT CCA TGG TCT TCT GGC 3'
shRNA-human DGKZeta sense	5' CCG GCT ATG TGA CTG AGA TCG CAA ACT CGA GTT TGC GAT CTC AGT CAC ATA GTT TTT 3'
shRNA-human DGKZeta antisense	5' AAT TAA AAA CTA TGT GAC TGA GAT CGC AAA CTC GAG TTT GCG ATC TCA GTC ACA TAG 3'
shRNASeq primer	5' GGC AGG GAT ATT CAC CAT TAT CGT TTC AGA 3'

9. Generation of viral particles and transduction

HEK293T cells were transfected with the appropriate retro- or lentiviral vector through DNA coprecipitation with calcium phosphate ($(Ca_3(PO_4)_2$) and the appropriate viral envelopes. DNA was used at a proportion of 2.5 μg/10^6 packaging cells, which were grown in 10 cm^2 plates. After 16 h of transfection, cell medium was replaced by that of the transduced cells and incubated for 24 h, after which the medium containing the viral particles was collected and centrifuged (1500 xg, 5 min, 4ºC). The remaining supernatant was filtered through a low-protein binding 45 μm filter and added to the cells at a 1:1 ratio with normal growth medium. Selection was started 48 h after infection (G418, 400 μg/mL, 7 days). MDA-MB-231-shRNA-control, -shRNA-DGKα, and -shRNA-DGKζ cell lines were derived from parental MDA-MB-231 by infecting the pSUPER.retro version of the indicated shRNA sequences. Establishment of stable and inducible MDA-MB-231-derived cell lines for DGK knockdown was achieved by infecting the pLKO-Tet-on-neo with the corresponding shRNA sequences.

10. Lipid kinase activity assays

10.1. DGK assay

DGK activity was measured using 1,2-dioctanoyl (C8)- or 1,2-dioleoyl (C18:1)-DAG as the substrate. To measure total kinase activity, cells were lysed by sonication in 50 mM Tris-HCl pH 7.5, with protease and phosphatase inhibitors. Cell lysates were centrifuged (12000 xg, 15 min, 4ºC). Whole cell extract supernatant (20 μg), membrane fraction or phosphotyrosine immunoprecipitates (obtained as described above) were mixed with lipid micelles containing the substrate.

Lipid micelles were prepared by sonication of the lipid in 50 mM Tris-HCl pH 7.5 (10 min, room temperature). The final concentration of both lipids in the assay was 0.5 mM (C8-DAG) and 2 mM (C18:1-DAG). When C18:1-DAG was the substrate, PtdSer was included in the micelles at the same molar ratio. The reaction was initiated by addition of reaction mix (1 mM ATP, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT and 10 μCi [γ-^{32}P]-ATP) and incubated (10 min, 25ºC) in a final volume of 50 μL. The reaction was terminated by addition of 1 N HCl. Lipids were extracted after addition of $CHCl_3$/MeOH (2:1, v/v). The organic layer was recovered, dried and applied to silica gel TLC plates (Whatman), which were developed in a $CHCl_3$/MeOH/4 M NH_4OH solvent system (9:7:2, v/v/v), dried, and autoradiographed. A non-radioactive standard of the reaction product was used to identify the lipid of interest.

10.2. PI3K assay

PI3K activity was measured in phosphotyrosine immunoprecipitates, obtained as described above, using PtdIns as substrate. PtdIns-containing micelles were prepared by sonication of the lipid in 10 mM Hepes pH 7.0 (5 min, room temperature). The final concentration of the lipid in the assay was 0.2 mM. The reaction was initiated by addition of reaction mix (20 μM ATP, 10 mM $MgCl_2$ and 10 μCi [γ-^{32}P]-ATP) and incubated (30 min, 25ºC) in a final volume of 50 μL. The reaction was terminated by addition of 1 N HCl. Lipids were extracted after addition of $CHCl_3$/MeOH (1:1, v/v). The organic layer was recovered, dried and applied to silica gel TLC plates (Whatman), which were developed in a $CHCl_3$/MeOH/4 M NH_4OH solvent system (9:7:2, v/v/v), dried, and autoradiographed. A non-radioactive standard of the reaction product was used to identify the lipid of interest.

11. Cell lipid extraction

Cell lipids were extracted by a modification of the method of Bligh and Dyer (Bligh and Dyer, 1959). Prior to extraction, an aliquot was stored for protein determination. Briefly, cells were rinsed thrice in cold PBS and resuspended in 1 mL of PBS; 4 mL of $CHCl_3$/MeOH/12 N HCl (50:100:1, v/v/v) were then added, and organic and aqueous phases separated by centrifugation (1500 xg, 10 min). The upper aqueous phase was further washed with $CHCl_3$. The recovered organic phases containing the lipids were mixed and dried under a nitrogen stream.

12. DAG level measurement

DAG levels were measured as described (Preiss et al., 1986). Dried lipids were solubilized in 20 μL of a 7.5% octyl-β-D-glucopiranoside and 25 mM dioleoylphosphatidylglycerol solution, sonicated (15 sec, room temperature) and incubated (10 min, room temperature). Resuspended lipids were then mixed with 70 μL of reaction mix (50 mM imidazole-HCl pH 6.6, 50 mM LiCl, 12.5 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT and 5 μg purified *Escherichia coli* DGK). The reaction was initiated by addition of [γ-^{32}P]-ATP (20 μCi) and allowed to proceed for 30 min. The reaction was terminated by addition of 1 N HCl. Lipids were extracted after addition of $CHCl_3$/MeOH (2:1, v/v). The organic layer was recovered, dried and applied to silica gel TLC plates, which were developed in a $CHCl_3$/acetone/MeOH/AcOH/H_2O solvent system (10:4:3:2:1, v/v/v/v/v), dried, and autoradiographed. Data were normalized by expressing the degree of DAG radiolabeling (phosphorimager pixels) per nmol of phospholipid phosphate.

Phospholid-associated phosphate was determined as described (Flores et al., 1996). Briefly, dried lipids were hydrolyzed in 70% perchloric acid (30 min, 180ºC). Samples were cooled and mixed with ammonium molybdate (3 mM final concentration) and ascorbic acid (72 mM final concentration), after which they were incubated (15 min, 56ºC), and absorbance measured at 690 nm.

13. *In vivo* radiolabeling of phospholipids

This assay was performed in collaboration with Dr. David Jones (Paterson Institute for Cancer Research, University of Manchester, Manchester, UK). MDA-MB-231 cells were seeded in 6-well plates. After 24 h, they were treated with DMSO, R59949, rapamycin or R59949 plus rapamycin for a further 24 h, washed three times with phosphate- and serum-free DMEM before reincubating with phosphate- and serum-free DMEM containing 300μCi/mL ^{32}P-orthophosphate and drugs for 1 h. Thereafter, cell lipids were extracted as described above. The recovered organic phase containing the lipids was dried under nitrogen. Of the total lipid extract, 20% was analyzed by TLC to determine PtdIns-4-phosphate and $PtdInsP_2$ radiolabeling. The remainder, 80%, was used to calculate the total phospholipid mass (as nmoles of phospholipid phosphate). Data were normalized by expressing the degree of PtdIns-4-phosphate and $PtdInsP_2$ radiolabeling (phosphorimager pixels) per nmol of phospholipid phosphate. HPLC was

used to verify the PtdIns-4-phosphate and $PtdInsP_2$ radiolabeling data using a previously published method (Jones et al., 1999).

14. Cell cycle protocol and analysis by flow cytometry

Cells were seeded at a confluence of 5 x 10^3 cells/cm^2, incubated 24 h, and then synchronized by serum starvation for 20 h. After this treatment, approximately 75% of the population was in G0-G1. Cell cycle entrance was induced by addition of FBS to a final concentration of 10%. Indicated drugs or vehicle were added 8 h before serum addition. At different points, cells were washed in PBS and trypsinized. Approximately 10^6 cells were analyzed by propidium iodide staining in an Epics XL-MCL (Beckman Coulter) cell sorter for cell cycle phase distribution. Cell cycle phases were adjusted with WinCycle software (Phoenix Flow Systems).

15. Analysis of apoptosis by flow cytometry

Cells (10^4 cells/cm^2) were seeded in 10-cm dishes, and after 24 h were serum-deprived. Drugs were then added at the indicated concentrations for 24 h. Cells were collected and stained with annexin V-FITC (Apoptosis Detection Kit I, BD Pharmingen) according to manufacturer's instructions. Propidium iodide (100 μg/mL) was added prior to cell sorting. The cell death profile was analyzed by flow cytometry in an in an Epics XL-MCL cell sorter.

16. Colony formation assays

Cells (5 x 10^2/well) were seeded in 12-well plates; after 48 h, drugs were added at indicated concentrations every 48 h for 7 days. Colonies and foci were stained with 0.1% crystal violet solution in 20% methanol. After extensive washing, crystal violet was dissolved in 10% acetic acid, and absorbance measured at 620 nm.

17. 3D cultures

MDA-MB-231 cells were cultured in a 3D matrix of collagen and laminin (Matrigel, BD Biosciences) as described (Debnath et al., 2003). Briefly, 50-60 μL Matrigel were added to each well of an 8-well chamber (1 cm^2 surface) and allowed to solidify (20 min, 37ºC). Cells were trypsinized and resuspended in assay medium (DMEM, 5% FBS, 2 mM L-Gln and 2.5% Matrigel); 1.5 x 10^3 cells/cm^2 were seeded. Assay

medium was changed every 48 h, and cells allowed to grow for 14 days. For RNA extraction, cells were seeded on 10 cm^2-plates and Matrigel culture resuspended directly into Trizol reagent.

18. Immunofluorescence

For immunofluorescence, cells were plated on gelatin-coated slides and, after the indicated treatments, were fixed in 4% paraformaldehyde (10 min, room temperature). The cells were permeabilized with 0.3% Triton X-100 (10 min) and blocked with 10% goat serum in TBS (30 min, room temperature). For GM130 staining, cells were permeabilized with 0.5% saponin. Cells were incubated with the appropriate primary antibody in incubation buffer (1% goat serum in TBS; 1 h, 37ºC or overnight, 4ºC) in a humidified chamber. Secondary antibodies were added to samples and incubated (1 h, room temperature). Finally, samples were incubated with DAPI or rhodamine-phalloidin (1:1000, 10 min, room temperature) and mounted with Aqua Poly/Mount (Polysciences). Cells were visualized under a Leica TCS SP5 confocal or a Leica DMI6000B fluorescence microscope.

19. Immunohistochemistry

Tissue sections were formalin-fixed and paraffin-embedded. Sections were prepared for antibody staining as described (Carracedo et al., 2008). Briefly, following deparaffinization and hydration, antigen retrieval was performed in 12.5 mM sodium citrate pH 6.0 (30 min, 90ºC). Tissue sections were washed in TBSt (TBS, 0.1% Tween-20) and blocked with 10% goat serum (1 h, room temperature). Primary antibody was incubated (overnight, 4ºC), followed by incubation with HRP-coupled secondary antibody (Dako, 1:200, 2 h, room temperature). Staining was detected with the DAB Peroxidase Substrate Kit (Vector Laboratories), and slides were counterstained with hematoxylin (Fisher Scientific) and mounted in Permount (EMS).

20. Cell sorting isolation of murine mammary gland subpopulations

The third and fourth mammary glands were remove from virgin female mice aged 8 to 25 weeks, after which they were dissociated (16 h, 37ºC) in DMEM/F12 (1:1, v/v) containing collagenase/hyalurodinase (Stem Cell Technologies). A single mammary cell suspension was obtained after sequential treatment with 0.25% trypsin and

dispase/DNase solution (2 min, room temperature), followed by filtration through a 40 μm cell strainer. The remaining cells were resuspended to a final concentration of 10^7 cells/mL and stained for fluorescence-activated cell sorting (FACS).

For FACS staining, cells were pre-blocked in 10% normal rat serum (10 min, 4ºC) and washed. Cells were then stained (10 min, 4ºC) with the following surface markers: CD49f-AF488, CD49b-PE, EpCAM-AF647, Sca1-PECy7 CD45-biotin, CD31-biotin, Ter119-biotin and BP1-biotin. Biotin-coupled antibodies, which bind to non-epithelial lineages, were detected with streptavidin APC-Cy7. Cells were finally stained with DAPI. Mammary epithelial subpopulations were sorted as indicated.

Cell type	Marker			
	CD49f	EpCAM	CD49b	Sca-1
Basal	High	Low		
Luminal	Medium	High		
Non-clonogenic			-	+
Double positive progenitor			+	+
Alveolar progenitor			+	-

RNA from sorted populations was extracted with the PicoPure RNA Isolation kit (MDS Analytical Technologies) according to the manufacturer's protocol, and used for qPCR analysis as described below.

21. RT-PCR

cDNA was synthesized from cell culture-extracted RNA (1 μg) after treatment with DNase (Invitrogen), and retrotranscribed with Oligo dT (Invitrogen) and the SuperScript II Reverse Transcriptase (Invitrogen) according to manufacturer's indications in a final volume of 20 μL.

PCR reactions were performed with 2 μL cDNA and appropriate primers. PCR conditions were: 95ºC for 2 min, 30 cycles at 95ºC for 30 s, 60ºC for 30 s and 72ºC for 1 min, and a final extension of 72ºC for 8 min. PCR products were resolved by 1.5% agarose gel electrophoresis, stained with ethidium bromide and documented with the GelDoc system (Bio-Rad Laboratories). Sequences of primers used are listed below:

Gene	Forward primer 5'	Reverse primer 3'
DGKα	5' CAA TCA CAT CTG TGG GTG CGA GGA 3'	5' TTC CCG CCA CTC TTA GGA TTG AC 3'
DGKβ	5' AAT GGA TCC TGA TCA TGA TGG AAC CGT GT 3'	5' GAT GAA TTC CAC CAA ACA CAA TGC AGT CC 3'
DGKγ	5' CTC ATG GGC GTC CGC AAG CA 3'	5' CAT CCG GCA CCA CAG GCA GT 3'
DGKδ1	5' GTG AAA GGG CAC CTT GAA AAC GC 3'	5' CCC AGA CAG AGC CTC ACG GCA 3'

DGKδ2	5' GAA GCT CAT CCG CAA GGT GTC CAC G 3'	5' ATT GCA GTA GGT CGG CCT CGC 3'
DGKη	5' GTT GCT GCT TGG CTG GAT CTG CT 3'	5' GCC ACA GAG GTG CTT CAG TGG TG 3'
DGKκ	5' GAT GAA CAC CCA GGG CAA TAC AAT AG 3'	5' ACC ATC AAT GGT TAT CAT CAC CTC ATG 3'
DGKε	5' AGG GCC CTG CAC TGT CAC CA 3'	5' AGG GGC ATG CTG CAG AGG AT 3'
DGKζ	5' CGC CTG CCA TGG AGA CTT TCT TTA G 3'	5' TTC ATG CCC ACA AGC CCG GTC 3'
DGKι	5' AGG GGA TCC CAG GAG GGG AAA TGT AAG CA 3'	5' CAA GAA TTC GCA AGG GTT TCA TGA GAG GA 3'
DGKθ	5' ATG GGA TCC AGG ATC ACG ACA CCC ATC AC 3'	5' ACG GAA TTC CGG AAC TGG CTT CTT CTC AC 3'
β-actin	5' GGC ACC ACA CCT TCT ACA ATG 3'	5' GTG GTG GTG AAG CTG TAG CC 3'

22. Quantitative RT-PCR

qPCR reactions were carried with the Power SYBR Green PCR Master Mix (Applied Biosystems) in a final volume of 8 μL. GAPDH (human) or RPL3A (mouse) were used as controls. . The primers used are detailed below:

Gene	Species	Forward primer 5'	Reverse primer 3'
SREBP-1	**Human**	5' TCA GCG AGG CGG CTT TGG AGC AG 3'	5' GAG TCT GCC TTG ATG AAG TG 3'
SREBP-2	**Human**	5' GGA TCA TCC AGC AGC CTT TGA 3'	5' ACC GGG ACC TGC TGC ACC TGT 3'
FASN	**Human**	5' GAA ACT GCA GGA GCT GTC 3'	5' CAC GGA GTT GAG CCG CAT 3'
ACC	**Human**	5' GCC TCT TCC TGA CAA ACG AG 3'	5' GAC TGC CGA AAC ATC TCT G 3'
SCD1	**Human**	5' CTA CAA ACC TGG CTT GCT GA 3'	5' CCA GCC AGG TGG CAT TAA 3'
HMGCR	**Human**	5' CTT GTT GAA TGT CTT GTG ATT G 3'	5' CTC TAA TAC CAA GGA CAC 3'
DGKα	**Human**	5' CAA TCA CAT CTG TGG GTG CGA GGA 3'	5' TTC CCG CCA CTC TTA GGA TTG AC 3'
GAPDH	**Human**	5' ACA GCC TCA AGA TCA TCA GCA A 3'	5' ATG GCA TGG ACT GTG GTC ATG 3'
DGKα	**Mouse**	5' GTC TAC TGC TAC TTC ACC CTC C 3'	5' CTT CCG TGC TAT CCA GGA TCC 3'
DGKα promoter 1	**Mouse**	5' CTC ACA CCA CCA TGC TGC TTC AAG AGT CCT G 3'	5' GGC CCT TCT CTT TGG CCA TCC TTC TTC TC 3'
DGKα promoter 2	**Mouse**	5' GGC CGC GTC TTC CTA ACC CAG TCC CCT CC 3'	5' GGC CCT TCT CTT TGG CCA TCC TTC TTC TC 3'
RPL3A	**Mouse**	5' TAA AGA CTG GAG ACA AGG TG 3'	5' GTG TAC TCA GTC TCC ACA GA 3'

PCR conditions were as follows: 50ºC for 10 min, 95ºC for 2 min, 40 cycles at 95ºC 15 s and 60ºC 1 min. Reactions were run with the Applied Biosystems 7900HT system. Samples for each experimental condition were run in triplicate. The relative expression of each gene was calculated from the fluorescence increase data *vs*. cycle number, from

which the *Ct* value is obtained; after correction for control amplification, the Δ*Ct* is obtained. Finally, the sample is compared with a reference sample, and the relative expression of the gene is calculated by applying the equation $2^{-(\Delta\Delta Ct)}$.

23. Induction of subcutaneous tumors with the MDA-MB-231 cell line and *in vivo* pharmacological treatment

All mouse work was done in accordance with a protocol approved by the CNB/CSIC Ethical Committee for Animal Experimentation (CEEA-CNB). MDA-MB-231-shRNA-control, -shRNA-DGKα, and -shRNA-DGKζ cells (1.5 x 10^6) were injected subcutaneously in the flank of female BALB/c SCID mice aged 6-8 weeks. Tumor growth was monitored every two days and volume estimated according to the formula: volume = (a^2 x b)/2, where a = tumor width and b = tumor length in millimeters. At the end of experiment (when control tumors reached 1 cm^3, ~30 days), tumors were weighed after sacrifice of the mouse and extraction of the tumor. Tumors were then fixed overnight in paraformaldehyde, followed by dehydration in graded ethanol and embedded in paraffin for immunohistochemical studies.

To assay the effect of R59949 and rapamycin on *in vivo* tumor growth, MDA-MB-231 parental cells (1.0 x 10^6) were injected subcutaneously in the flank of female BALB/c SCID mice aged 6-8 weeks. Pharmacological treatment was initiated when tumors reached at least 150 mm^3 (~15 days). Rapamycin and R59949 were administered intraperitoneally or subcutaneously, respectively, at a dose of 10 mg/kg every 48 h for 10 days. Mice were sacrificed 48 h after the last dose, and tumors processed as above.

24. Analysis of DGKα and DGKζ expression in human breast samples

DGK expression was analyzed in normal and tumor-derived human breast tissue from FFPE biopsies stored at the Hospital Clínico Universitario, Valencia, Spain. We included five normal breast and nine tumor-derived samples. Among the tumor-derived samples, five were classified as luminal, two as triple negative and two as HER2+. mRNA was extracted from samples with the High Pure FFPE RNA microkit (Roche) according to the manufacturer's protocol. Probes to DGKα, DGKζ and TBP (used as

control) were purchased from Applied Biosystems. qPCR conditions were as described above.

25. Statistical analysis

Data are represented as the mean ± SEM. Statistical analysis was done with the GraphPad Prism 5 Software. Student's *t*-test was performed to compare data. When the variance was significantly different, as analyzed with F-test, Welch's correction was applied. When the samples did not fit to normality, as tested with the Kolmogorov-Sminorv test, the Mann-Whitney test was used. When the p-value < 0.05, differences were considered statistically significant (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

RESULTS

1. ANALYSIS OF DGKα AND DGKζ ISOFORM EXPRESSION AND ACTIVITY IN BREAST CANCER AND NORMAL MAMMARY GLAND AND CORRELATION WITH THAT OF THEIR REGULATORS

1.1. Determination of DGK expression and activity in breast cancer cell lines

The DGK family comprises 10 isoforms, classified in five subgroups based on their domain structure (Merida et al., 2008). Its expression is particularly enriched in the immune and the central nervous systems. Most isoforms are expressed in epithelial lineages. As there has been no analysis of DGK expression in the breast, our first aim was to identify which DGK isoforms are expressed in breast cancer and to compare DGK activity in normal and tumor cells. We selected a panel of breast cancer-derived cell lines that include different oncogenic alterations and are representative of the distinct breast cancer subtypes (for details see Material and Methods). As controls, we used two immortalized non-malignant cell lines of luminal (HB4a) and basal (MCF-10A) origin, which resemble the normal epithelium.

Breast cancer cell lines expressed at least five DGK isoforms

Our first approach to identify which DGK isoforms are expressed in breast-derived cell lines was to analyze their mRNA by RT-PCR. mRNA levels do not always correlate with protein expression, but the lack of antibodies to all DGK isoforms rendered this the only suitable option for this analysis.

RT-PCR analysis of five selected cell lines showed that at least five DGK isoforms were expressed in all cell lines. We identified expression of type I DGKα, type II DGKδ and DGKη, type III DGKε and type IV DGKζ. Type V DGKθ was observed only in basal-like cell lines (MCF-10A, MDA-MB-231 and MDA-MB-468). In additional, type IV DGKι was detected only in the $HER2^{+}$ cell line BT-474. Type I DGKγ expression was nearly undetectable, and we considered it negative. We did not detect type I DGKβ or type II DGKκ (**Figure 8A**).

DGK levels and activity are reduced in breast cancer-derived cell lines

We next evaluated DGKα and DGKζ protein levels in the breast cell line panel, as DGKα and DGKζ are the best-characterized isoforms. Several studies suggest that DGKα promotes cancer progression (Filigheddu et al., 2011), whereas array analyses indicate that DGKα acts as a tumor suppressor. DGKζ has not been related to cancer onset; however, its redundancy with DGKα and the fact that it is a major DAG metabolizer necessitate analysis of its function in cancer progression.

We first analyzed DGKα and DGKζ expression in untransformed cell lines of basal (MCF-10A) or luminal (HB4a) origin. We found no great differences in the expression of these DGK isoforms between these cell lines (**Figure 8B**). The MCF-10A cell line was used as an untransformed control. DGKα expression was diminished in all tumor-derived cell lines. This reduction was more prominent in those cell lines bearing oncogenic alterations that lead to constitutive activation of the PI3K-Akt axis, including mutation in the p110α PI3K catalytic subunit (MCF-7), lack of the PTEN phosphatase (MDA-MB-468) or HER2 overexpression (BT-474). DGKζ expression was higher in tumor lines than in untransformed cell lines, and was most strongly expressed in the MCF-10A cell line (**Figure 8C**).

To analyze DGK activity, we performed two types of assays in crude cell extracts. In the first, C8-DAG micelles (as substrate) were formed directly in kinase assay buffer. This assay allowed measurement of total DGK activity. If octyl glucoside (OGF) is added, DGKα activity is excluded; in these conditions, there are no differences in activity among cell lines, although $HER2^{+}$ cells showed the lowest activity. When DGK activity was measured without OGF addition, DGK activity paralleled DGKα levels; activity was higher in control untransformed cells and decreased in tumor cell lines. Of the transformed cell lines, those bearing mutations in the p110α PI3K catalytic subunit (MCF-7, T-47D) or overexpressing the HER2 receptor (BT-474) had the highest activity (**Figure 8D**).

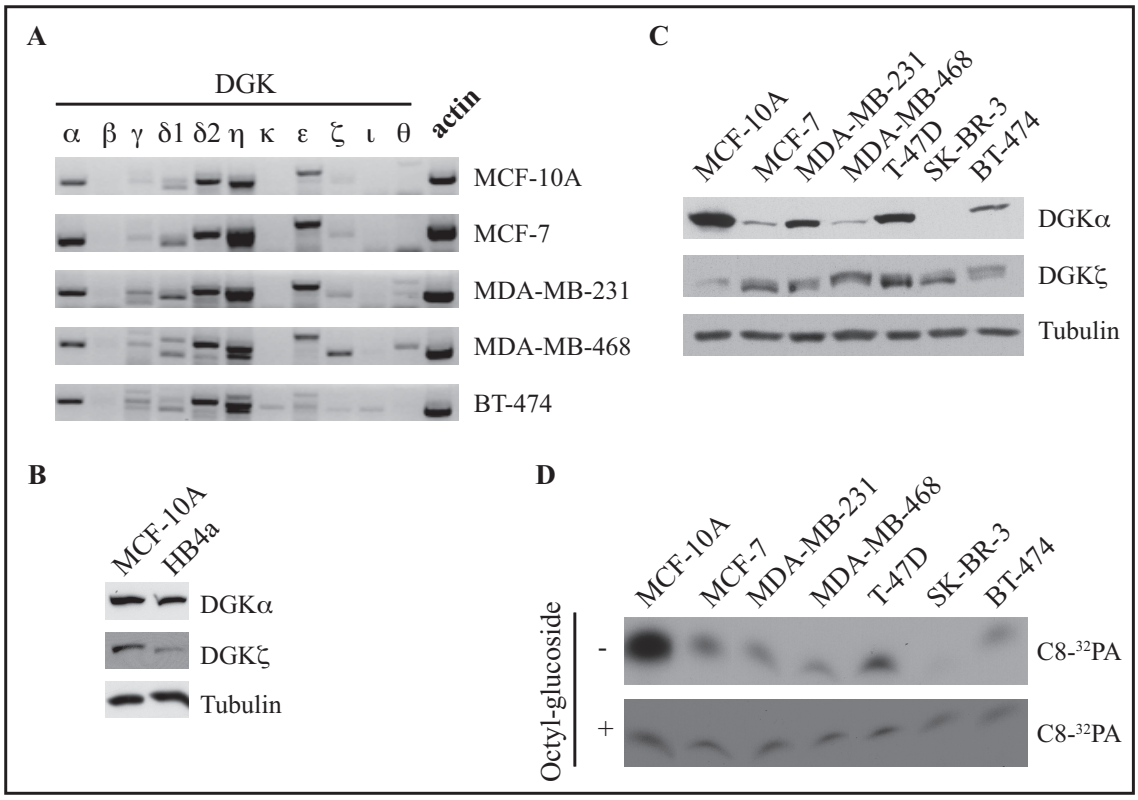


Figure 8. DGK expression and activity in the breast-derived cell lines used in this study. (A) DGK isoform expression in the indicated cell lines was determined by analyzing mRNA expression in RT-PCR. (B) Western blot comparison of DGKα and DGKζ in MCF-10A and HB4a cell lines. (C) Western blot analysis of DGKα and DGKζ protein levels. DGKα is expressed in the SK-BR-3 cell line, although at very low levels. (D) DGK activity assay in whole cell extracts of the different cell lines was measured using C8-DAG as substrate, alone (top) or with octyl glucoside (bottom). Each assay was performed at least three times. A representative image is shown.

DGK activity is coupled to the HER2 receptor in HER2$^+$ cell lines

The data above suggested that DGKα accounts for most of the changes in DGK activity observed in breast cancer cell lines. DGKα is regulated by tyrosine phosphorylation, which stabilizes the enzyme in membranes. We therefore measured DGK activity in phosphotyrosine immunoprecipitates. In these conditions, we observed similar activity in the untransformed cell line and the highly malignant MDA-MB-231 cell line. The highest activity was observed in tumor-derived cell lines that overexpress RTK (EGFR in MDA-MB-468; HER2 in BT-474) or RTK scaffold (IRS-1 in MCF-7; **Figure 9A**).

The BT-474 cell line, which overexpresses the HER2 RTK, showed the highest phosphotyrosine-coupled DGK activity. Some DGK isoforms are regulated downstream of SFK (Cutrupi et al., 2000), and the increase in DGK activity could therefore be a result of increased SFK activity downstream of the receptor. To

determine whether active DGK binds to the HER2 receptor, we immunoprecipitated HER2 from two overexpressing cell lines, BT-474 and SK-BR-3. As a negative control, we used the HER2-negative MCF-7 cell line. After incubation of HER2 precipitates with DAG and ATP, we detected a product that comigrated with PA, confirming that active DGK is directly coupled to this receptor (**Figure 9B**).

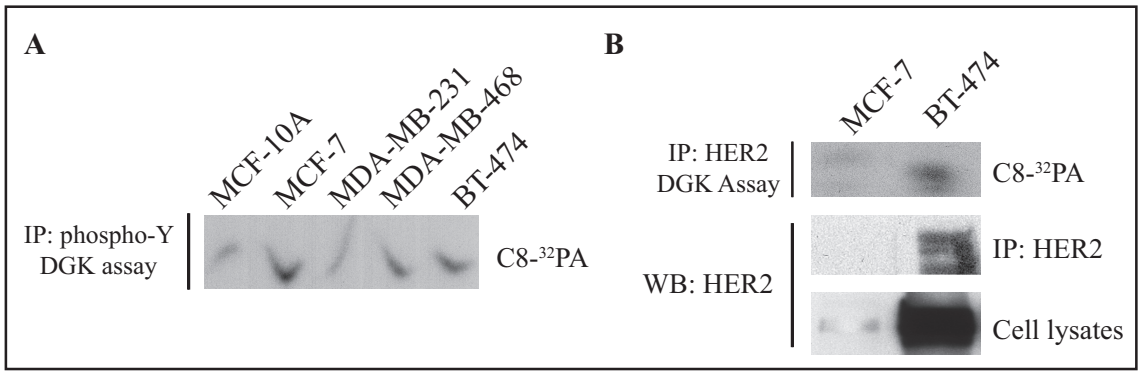


Figure 9. DGK activity is associated with phosphotyrosine and the HER2 receptor. (A) DGK activity was measured in phospho-Y immunoprecipitates from cell lines, using C8-DAG as substrate. (B) HER2 was immunoprecipitated and DGK activity assayed (top). Immunoprecipitation (center) and expression of HER2 (bottom) is shown in the cell lines used.

DGKα levels are regulated downstream of the PI3K pathway

We thus observed that DGKα levels were diminished in breast cancer-derived cell lines, and that this reduction was more prominent in cell lines with oncogenic alterations in the PI3K axis. A study from our laboratory suggests that through Akt-mediated FoxO inhibition, DGKα is negatively regulated at the transcriptional level downstream of the PI3K-Akt axis (Martínez-Moreno et al., under revision). There is increasing evidence of FoxO inactivation in tumors as a result of PI3K-Akt pathway hyperactivation. To assess whether lack of FoxO function due to oncogenic activation of the PI3K-Akt pathway correlated with changes in DGKα expression, we used RT-PCR to determine *DGKα* expression in breast cancer-derived cell lines showing aberrant PI3K pathway activation. As a control, we included the MCF-10A cell line, which has no alterations in the PI3K axis. *DGKα* gene expression was reduced in tumor cell lines compared to MCF-10A cells (**Figure 10A**). Western blot experiments indicated inverse correlation between FoxO phosphorylation and DGKα protein levels in tumor and control breast cells (**Figure 10B**). Even among tumor cells, variations in DGKα protein levels correlated to gene expression detected in RT-PCR and the degree of FoxO phosphorylation. This is the case of the PTEN-deficient MDA-MB-468 cells, which showed the lowest DGKα and the highest FoxO1 phosphorylation levels.

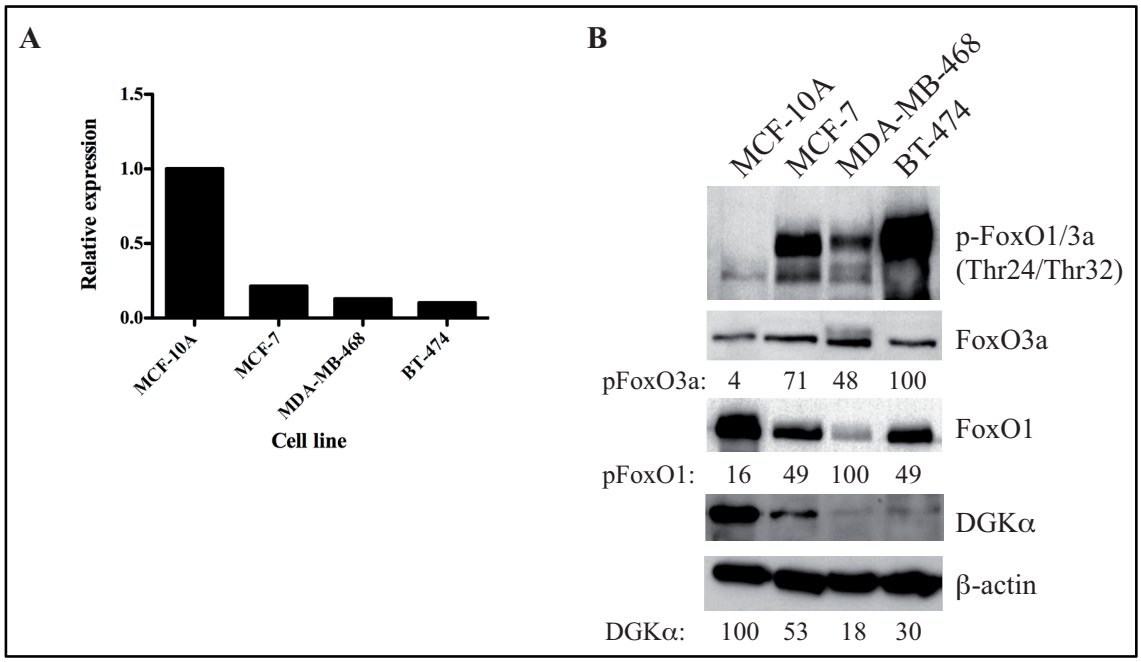


Figure 10. DGKα expression correlates inversely with PI3K-Akt pathway activation in breast cancer cell lines. (A) RT-qPCR quantification of *DGKα* mRNA levels in the cell lines compared to MCF-10A. (B) Lysates of cell lines were resolved by SDS-PAGE; DGKα protein levels and FoxO1 and FoxO3 phosphorylation status were determined. Blots were quantified with ImageJ. Relative DGKα expression and FoxO phosphorylation are indicated (bottom). DGKα expression by MCF-10A cells = 100; pFoxO3a by BT474 cells = 100; pFoxO1 by MDA-MB-468 cells = 100.

1.2. Analysis of DGKα expression in the normal mouse mammary gland

The mammary gland normally develops during puberty, which in mice takes place around 10-11 weeks after birth. Mammary gland development involves cycles of ductal elongation, side branching and involution. This is a highly proliferative process under hormone-dependent control (Brisken and O'Malley, 2010). Microarray analyses place *DGKα* as a quiescence- and stemness-related gene (Merida et al., 2009). We used qPCR to determine which mammary cells expressed *DGKα*, and compared the expression of this isoform before and after the onset of puberty.

Enzymatic digestion of the mammary gland, followed by fluorescence-activated cell sorting (FACS) based on staining of the epithelial cell adhesion molecule (EpCAM) and α6-integrin (also referred as CD49f) allows the isolation of two populations (Stingl et al., 2006), basal or myoepithelial cells ($EpCAM^{low}$ $CD49f^{high}$) and luminal cells ($EpCAM^{high}$ $CD49f^{medium}$). We observed that prior to (8 weeks) and after puberty (>11 weeks), *DGKα* was expressed mainly in the luminal compartment. At the onset of

puberty, expression was higher in basal-derived cells, which might correlate with increased luminal cell proliferation at this stage (**Figure 11A**).

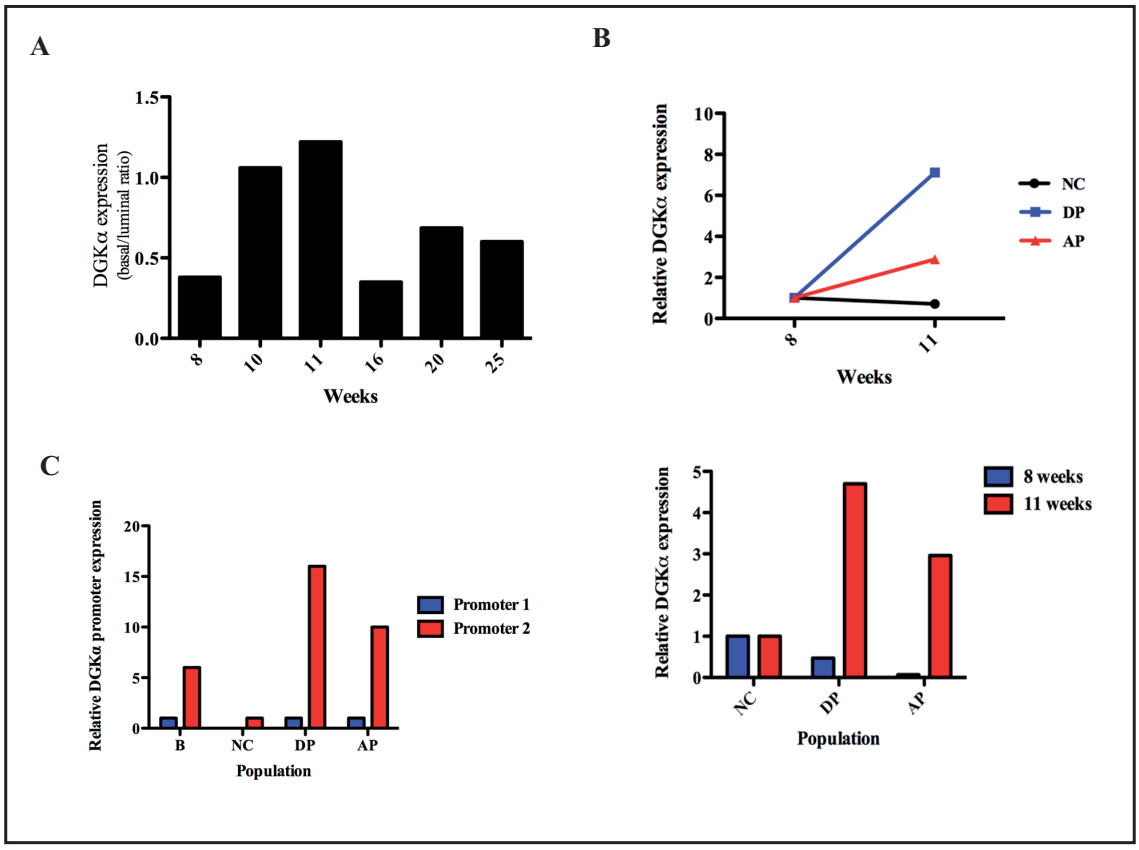


Figure 11. qPCR analysis of DGKα expression in the normal mouse mammary gland. (A) Relative *DGKα* expression in luminal and basal mammary cells during post-birth development. Data are presented as the basal-luminal ratio. (B) Analysis of relative *DGKα* expression in luminal subpopulations; variation in each subpopulation at the onset of puberty (top) and comparison of subpopulations before and during puberty (bottom). (C) Relative expression of *DGKα* promoters in the distinct mammary cell populations. Each assay was performed at least twice.

The luminal cell population can be further divided into three subpopulations based on expression of Sca1 and α2-integrin (also termed CD49b) (Shehata et al., personal communication): alveolar progenitor ($Sca1^-$ $CD49b^+$), double-positive or ER^+ progenitor ($Sca1^+$ $CD49b^+$), and non-clonogenic or luminal differentiated cells ($Sca1^+$ $CD49b^-$). We compared *DGKα* expression in these populations before and during puberty. *DGKα* mRNA levels increased at the onset of puberty in both progenitor populations, whereas its levels remained constant in the non-clonogenic cells (**Figure 11B, top**). Whereas *DGKα* expression was higher before puberty (8 weeks) in the non-clonogenic subpopulation, this pattern changed at 11 weeks, suggesting that *DGKα* is necessary for mammary cell differentiation programs (**Figure 11B, bottom**).

Expression of the *DGKα* gene is driven by the action of two promoters (Martínez-Moreno et al., under revision). We tested whether the expression of the two promoters was similar in mammary cells or if one was more prominent than the other. qPCR analysis demonstrated that most *DGKα* gene expression in mammary cells is due to promoter 2 activity, whereas expression of promoter 1 was barely detectable (**Figure 11C**). These data indicate that *DGKα* expression depends on the mammary gland proliferative-differentiation stage.

2. CONTRIBUTION OF DGKα AND DGKζ TO BREAST CANCER PROGRESSION

2.1. DAG consumption by DGKζ promotes SREBP-1 processing

To sustain proliferation, tumor cells must adjust their metabolism by increasing the flow through biosynthetic pathways. *De novo* lipid synthesis is normally increased in tumor cells, to generate the lipid membranes of daughter cells. Several signaling pathways that ultimately regulate the SREBP family of transcription factors control lipid biosynthesis. In addition, DAG and PA are central metabolites for lipid synthesis. We therefore tested whether DGK controlled the signaling and metabolic inputs that underlie lipid synthesis control.

DAG levels increase after DGK inhibition and DAG accumulates in perinuclear regions in the MDA-MB-231 cell line

To address DGK function in the regulation of tumor lipid metabolism, we used a broad spectrum DGK inhibitor, 3-[2-(4[bis-(4-fluorophenyl)methylene]-1-piperidinyl)ethyl]-2, 3-dihydro-2-thioxo-4(1H) quinazolinone (DGK inhibitor II or R59949). This drug is more potent than DGK inhibitor I, and blocks activity by binding to the DGK catalytic domain (Jiang et al., 2000). Although R59949 is reportedly a selective inhibitor of Ca^{2+}-dependent type I DGK isoforms *in vitro* (Jiang et al., 2000), it also inhibits other DGK classes *in vivo* (Avila-Flores et al., 2005; Chibalin et al., 2008).

Total DGK activity was assayed in lysates of breast cancer-derived MDA-MB-231 cells treated with various concentrations of R59949. The inhibitor reduced DGK activity by 50% at a 30 μM concentration (**Figure 12A**), which we established as the

IC_{50}. Since DGK regulates DAG levels, we measured lipid levels after prolonged (24 h) cell treatment with R59949. Consistent with the reduction in DGK activity, total DAG mass was increased by 40% compared to vehicle-treated cells (**Figure 12B**).

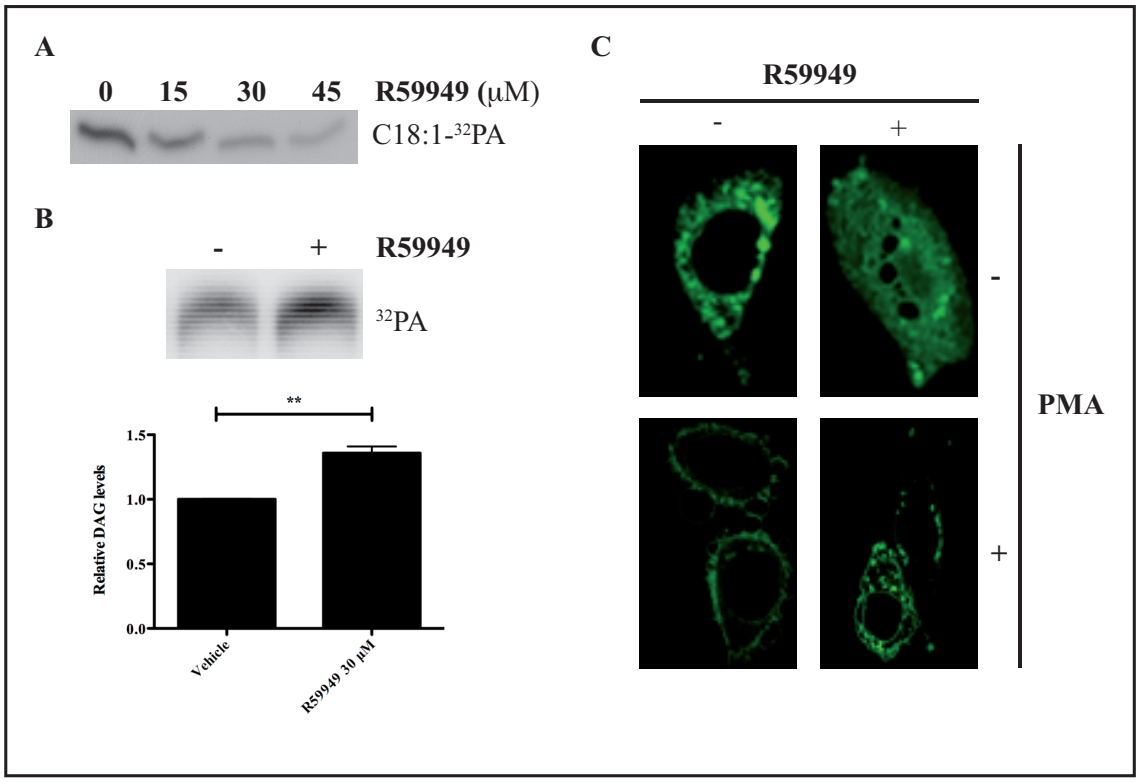


Figure 12. DGK inhibition leads to chronic DAG increase in intracellular membranes. (A) MDA-MB-231 cells in exponential growth were treated with DMSO or the indicated doses of the DGK inhibitor R59949 (24 h) and total DGK activity was evaluated. (B) Total cellular DAG was determined using an *in vitro* radioenzymatic assay. A representative autoradiogram is shown (mean ± SEM; n ≥3). (C, top) DAG localization in DMSO- or R59949-treated MDA-MB-231 cells was determined after transfection of the GFP-PKCθ C1b domain. (C, bottom). Response of the PKCθ C1b domain to PMA was evaluated in the same conditions. Student's *t*-test; **p <0.01.

DAG can be generated in distinct cell compartments from several sources, allowing the co-existence of different DAG pools, each of which has specific signaling and metabolic roles. To study the sites at which DAG accumulates, we used the DAG sensor PKCθ C1b domain (Carrasco and Merida, 2004) and tracked its location *in vivo* after DGK inhibition. Transfection of MDA-MB-231 cells with the GFP-fused PKCθ C1b domain showed that DAG accumulated mainly in a perinuclear region, whereas it was almost undetectable in the plasma membrane (**Figure 12C, left column, top**). To confirm that the PKCθ C1b domain responded to DAG levels, we used the non-permeable DAG analogue PMA (phorbol-12-myristate-13-acetate), which caused C1b translocation to the plasma membrane in vehicle-treated cells (**Figure 12C, left**

column, bottom). Prolonged R59949 treatment led to the appearance of a signal in vesicles, that did not mimick PMA addition, while perinuclear staining was maintained (**Figure 12C, right column, top**). In R59949-treated cells, PMA addition decreased the C1b signal in the perinuclear region, accumulated it at the plasma membrane, and was unaltered in the vesicles (**Figure 12C, right column, bottom**). These results indicate that DAG accumulates in perinuclear regions and in vesicles, and not in the plasma membrane, when DGK activity is inhibited.

DGK inhibition sustains PKD activation and affects Golgi apparatus structure

DAG activates classical and novel PKC. To test the effect of DAG increase, we treated MDA-MB-231 cells with R59949 for different times and measured phospho-PKC substrates to analyze PKC activation; we found a slight increase in PKC activity as a result of DGK inhibition (**Figure 13A**). In addition to activate PKC, DAG also activates PKD. To be fully active, PKD must be phosphorylated at two sites, one formed by two residues within its activation loop domain (Ser744/8 for PKD1) whose PKC-dependent phosphorylation primes and relocates PKD, and the other comprised of a carboxy-terminal residue (Ser916 for PKD1), the result of autophosphorylation. PKC-dependent phosphorylation of PKD increased shortly after R59949 addition and was sustained for the duration of treatment (**Figure 13B**).

Once activated, PKD localizes to the Golgi, where it promotes PtdIns-4-phosphate generation through activation of PI4KIIIβ (Yeaman et al., 2004). PtdIns-4-phosphate is sensed by lipid transporters with a PH domain; we used the GFP-tagged PH domain of the ceramide transfer protein CERT to track PtdIns-4-phosphate accumulation. In control cells, this lipid showed perinuclear accumulation, consistent with its Golgi location (**Figure 13C**), whereas in R59949-treated cells, perinuclear staining was lost and a vesicular pattern was observed throughout the cell (**Figure 13C**). This is consistent with reports indicating that prolonged PKD activation leads to Golgi vesiculation. To confirm this alteration in Golgi structure, we performed indirect immunofluorescence for the 130 kDa Golgi matrix protein (GM130). DGK inhibition modified GM130 staining in a time-dependent manner, from a compact perinuclear to a multivesicular cytosolic signal (**Figure 13D, E**), reminiscent of the pattern for the PKCθ C1b domain. This correlates with previous reports that show a critical contribution of DAG to maintain a correct lipid distribution in inner membranes, and thus the structure of organelles such as the Golgi (Baron and Malhotra, 2002).

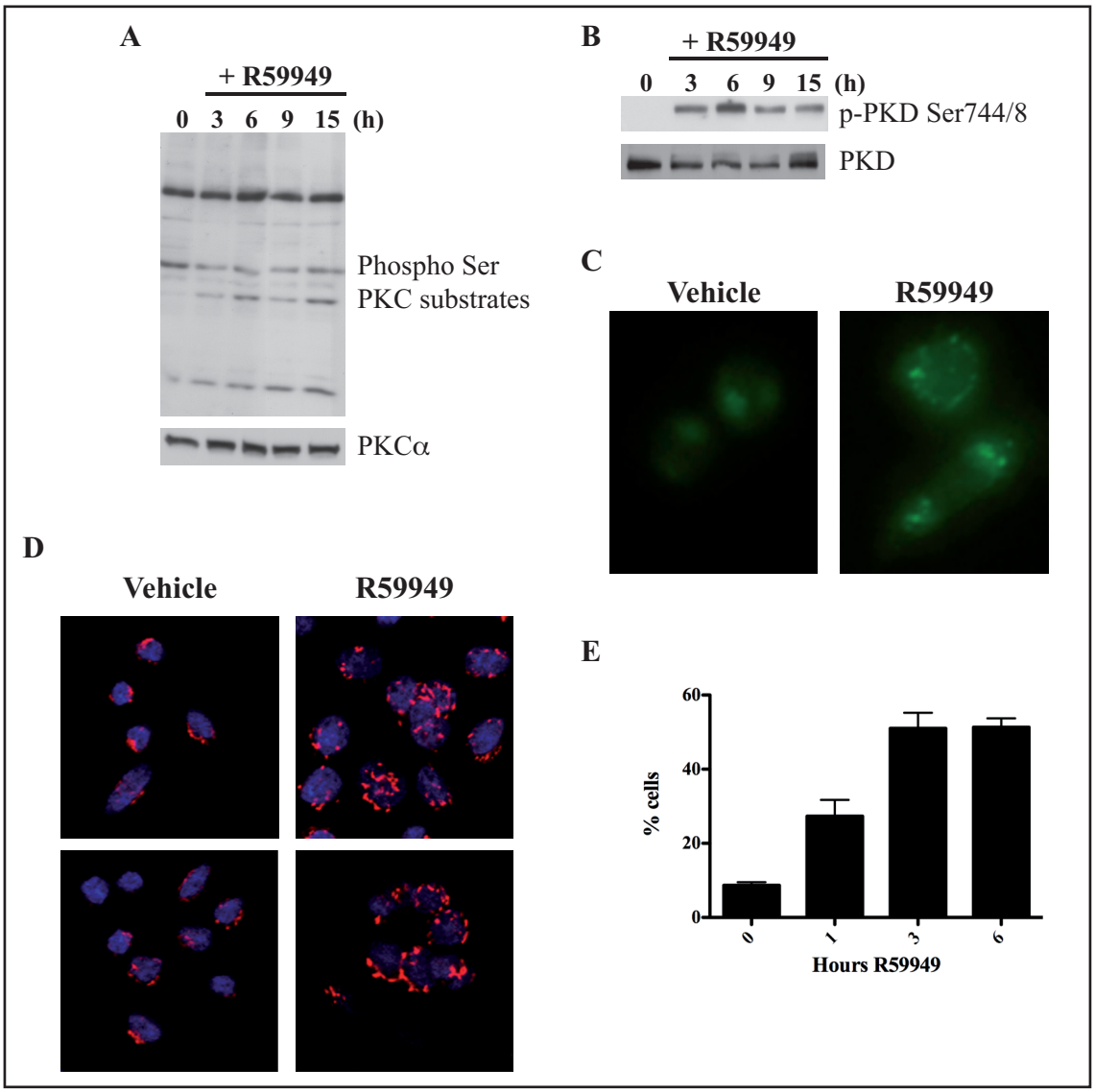


Figure 13. Inhibition of DGK sustains PKD activation and alters Golgi structure. MDA-MB-231 cells in exponential growth were treated with R59949 (30 μM) for the times indicated. Overall PKC (A) and PKD (B) activation status were measured by western blot. (C) Fluorescence distribution of the Golgi lipid PtdIns-4-phosphate was evaluated in DMSO- or R59949-treated (6 h) MDA-MB-231 cells by localization of the GFP-tagged CERT PH domain. (D) Golgi integrity was evaluated by immunofluorescence of the GM130 protein in cells treated as in C). (E) Time-dependent quantification (% cells with vesiculated Golgi; mean ± SEM) of cells processed as in D) is shown for at least three fields each in three independent experiments.

Sustained inhibition of diacylglycerol kinase affects SREBP-1 processing

These findings showed that DGK control the specific activation of DAG effectors in inner cell compartments, and are implicated in maintaining adequate lipid balance and Golgi integrity. In addition to this signaling role, DAG is a biosynthetic precursor for several lipids. Since the SREBP-1 transcription factor is a master regulator of lipogenic enzyme expression that senses lipid levels and is processed at the Golgi, we tested whether DGK contributes to SREBP-1 processing. To evaluate the effect of DGK inhibition on total SREBP-1 levels, we treated MDA-MB-231 cells with R59949 for

various periods. Levels of the full-length SREBP-1 form were the first to be reduced, whereas levels of the processed, active form decreased at longer treatment times (**Figure 14A**). Since SREBP-1 controls the expression of its own promoter, we tested whether reduced SREBP-1 transcription activity was responsible for the early reduction in precursor levels. qPCR analysis of SREBP-1 transcription activity indicated that SREBP-1 target expression as well of the gene itself were significantly reduced when DGK activity was inhibited, even at treatment times at which levels of the active short form had not yet been affected (**Figure 14B**). Inhibition of DGK activity thus mainly affected SREBP-1 transcriptional activity.

SREBP-1 activity depends on its subcellular location. We used fractionation analysis of DGK-inhibited cells to assess whether the processed form enters the nucleus. DGK inhibition promoted a decrease in the full-length and intermediate forms of SREBP-1. In addition, processed SREBP-1 levels were lower in the nuclear compartment, as this form accumulated in the cytosolic fraction; DGK inhibition thus prevented cytosol-nucleus translocation of mature SREBP-1 (**Figure 14C**).

SREBP-1 translocation to the nucleus depends on its phosphorylation state, such that it remains cytosolic when phosphorylated (Li et al., 2011). We tested whether SREBP-1 phosphorylation could be regulated in a DGK-dependent manner. The processed SREBP-1 form has a consensus substrate sequence for PKC-PKD. Western blot with a phospho-PKC substrate antibody showed that processed SREBP-1 was phosphorylated in the basal state; phosphorylation increased when DGK was inhibited (**Figure 14D**). Treatment of cells with PKC and PKD inhibitors increased levels of both full-length and processed SREBP-1 (**Figure 14E**).

As DGK inhibition led to an increase in total DAG levels, we determined whether this was the cause of altered SREBP-1 activity and processing. In MDA-MB-231 cells treated with dioctanoyl-DAG, a non-metabolizable DAG form, we observed a slight increase in PKD activation, which correlated with reduced SREBP-1 levels (**Figure 14F**). In a DGK assay of DAG-treated cells, we detected an increase in DGK activity that was sensitive to R59949 treatment (not shown). Co-treatment with DAG and R59949 led to sustained PKD activation and early loss of SREBP-1 levels (**Figure 14F**). These experiments indicate that SREBP-1 activation is DAG-sensitive, and that by controlling the levels of this lipid, DGK contribute positively to SREBP-1 activity.

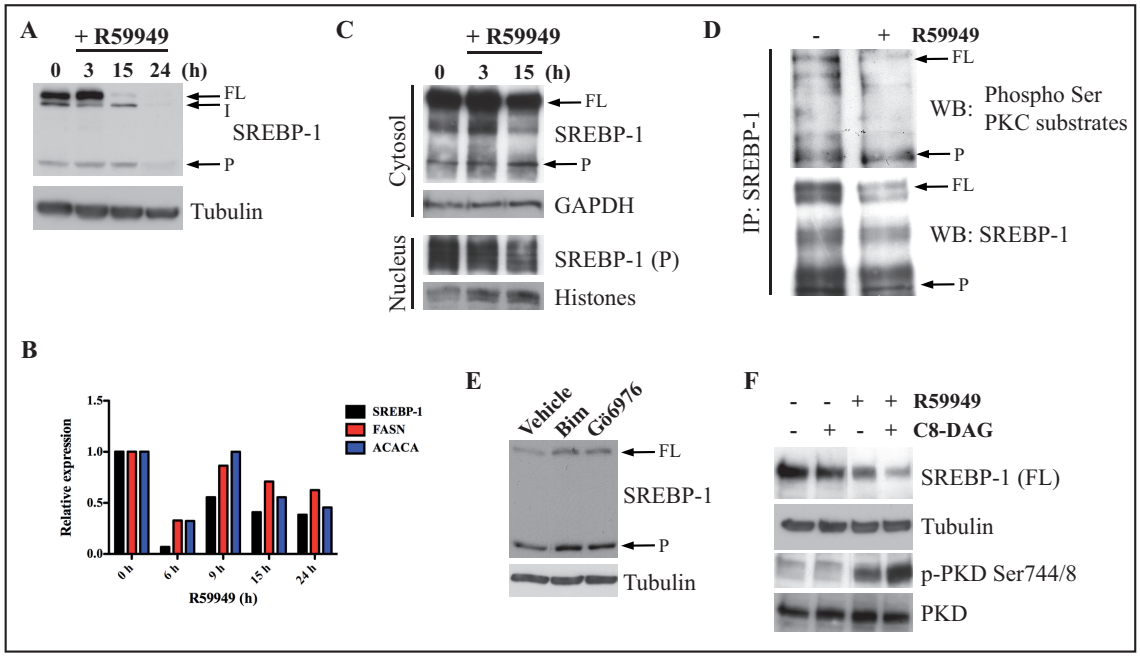


Figure 14. DGK contributes to SREBP-1 processing and transcription activity. (A) MDA-MB-231 cells in exponential growth were moved to medium containing 1% FBS, treated with R59949 (30 μM) for the times indicated, and SREBP-1 levels were determined by Western blot. (B) MDA-MB-231 cells were treated as in A) and mRNA levels measured by qRT-PCR. Expression shown is relative to untreated controls. (C) MDA-MB-231 cells were treated as in A), cytoplasmic and nuclear fractions were isolated, and levels of the indicated proteins analyzed by Western blot. (D) MDA-MB-231 cells were treated with R59949 (30 μM, 16 h) and SREBP-1 immunoprecipitated. PKC-mediated phosphorylation was analyzed by Western blot. (E) MDA-MB-231 cells were treated with bisindolmaleimide (Bim) or Gö6976 (both at 100 nM, 16 h) and SREBP-1 levels were analyzed by Western blot. (F) MDA-MB-231 cells were treated (3 h) with R59949 (30 μM) or C8-DAG (100 μM) and full-length SREBP-1 levels analyzed by Western blot. Data are representative of one experiment; $n \geq 3$.

Diacylglycerol kinase ζ is responsible for most DGK activity in breast cancer cells

The previous set of experiments suggested that the DGK pathway is a central regulator of DAG metabolism and signaling and therefore, that it controls SREBP-1 processing. Breast cancer-derived cell lines express various DGK isoforms. To identify the major DAG regulators in breast cancer cells, we focused on DGKα and ζ. DGKα is implicated in cancer survival and is the main isoform inhibited by R59949 (Jiang et al., 2000); DGKζ is regulated by PKC and its activity responds to DAG levels (Luo et al., 2003b) (Gharbi et al., 2011). We used established RNAi sequences to downregulate DGKα and ζ expression (Avila-Flores et al., 2005), and found a ~90% reduction in protein expression by each of these isoforms (**Figure 15A**). DGK activity assays of total cell extracts from RNAi-transfected cells indicated that whereas total DGK activity was unaffected by DGKα depletion, it decreased by ~50% when DGKζ levels were reduced (**Figure 15B**). We used the same assay to assess the contribution of other isoforms, after pretreating cells with several concentrations of R59949 (24 h).

Control cells showed a 50% reduction in DGK activity at the highest inhibitor dose, as for untreated DGKζ-depleted cells. When DGKζ-depleted cells were treated with R59949, however, we observed a further dose-dependent reduction in DGK activity, suggesting that a decrease in DGKζ permits R59949 access to other DGK isoforms that account for the remaining DGK activity in breast cancer cells (**Figure 15C**). DGKζ would thus be the principal regulator of DAG levels in these cells.

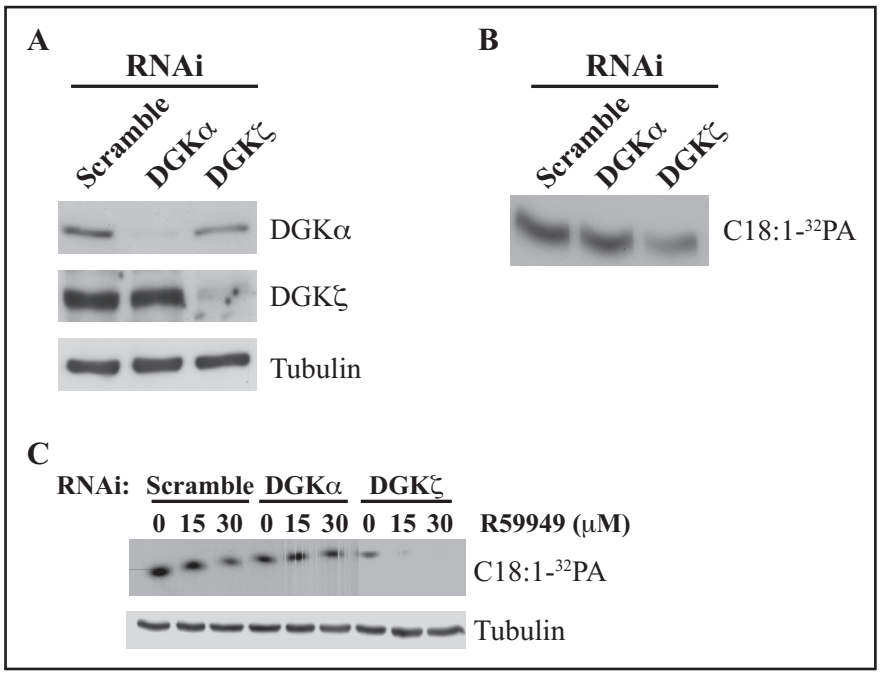


Figure 15. DGKζ is the major contributor to DGK activity in MDA-MB-231 cells. (A) DGKα and DGKζ levels in MDA-MB-231 cells transfected with siRNA for each isoform. (B) Total DGK activity was evaluated in MDA-MB-231 cells transfected as in A). (C) Total DGK activity in MDA-MB-231 cells transfected as in A) and treated with the indicated doses of R59949 (24 h). Data shown for one representative assay ($n \geq 3$).

Diacylglycerol kinase ζ is the main PKC-PKD regulator and is necessary to maintain SREBP-1 processing

Our results indicated that DGKζ is the main regulator of DAG levels in MDA-MB-231 cells. Abrogation of DGK activity led to a sustained increase in DAG levels that leads to alteration of DAG signaling effectors and lipid metabolism. In addition to DGKζ, we studied DGKα, which is involved in tyrosine kinase receptor signaling and might contribute to SREBP-1 activation.

To determine the isoform responsible for PKC and PKD regulation, we depleted each of these DGK isoforms in MDA-MB-231 cells and added R59949 to inhibit other DGK. This inhibition led to an increase in total PKC activity in control cells and in PKC phosphorylation at the activation loop and the C-terminal hydrophobic motif,

needed to maintain an active PKC conformation (**Figure 16A**). Depletion of DGKα or DGKζ increased PKC activity, which did not increase further after R59949 treatment. DGKα depletion increased PKC phosphorylation only at the activation loop, which is phosphorylated by PDK-1. In DGKζ-depleted cells, PKC phosphorylation increased not only at the activation loop, but also at the hydrophobic motif, assumed to be the result of autophosphorylation (**Figure 16A** (Cenni et al., 2002)).

PKD is activated by a combination of PKC triggering and DAG accumulation (Fu and Rubin, 2011). Addition of a DGK inhibitor or reduction of DGKα levels promoted the appearance of slow-migrating bands corresponding to phosphorylated states of PKD; this effect was more prominent in DGKζ-depleted cells, in which PKD migrated as a fully phosphorylated form (**Figure 16B**).

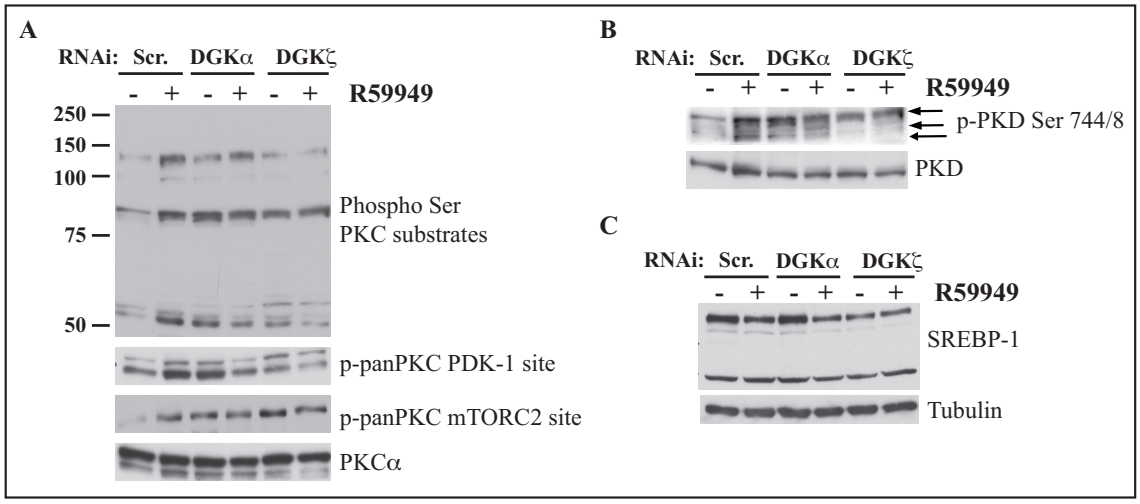


Figure 16. DGKζ depletion activates PKC-PKD signaling and abrogates SREBP-1 processing. DGKα and DGKζ expression was reduced in MDA-MB-231 cells by siRNA transfection. (A) Transfected cells were treated with R59949 (30 μM) or DMSO (16 h). PKC activity and phosphorylation status was evaluated by Western blot. (B) Western blot evaluation of PKD activation in cells treated as in A). (C) Western blot analysis of full-length and processed SREBP-1 levels in MDA-MB-231 with reduced DGKα or DGKζ levels. $n \geq 3$ for all experiments.

In summary, these data indicated that DGKζ is the main isoform implicated in the homeostatic regulation of DAG levels and the activation of its signaling effectors in MDA-MB-231 cells. By limiting DAG levels, DGKζ controls lipid metabolism through SREBP-1 regulation. Depletion of DGKζ in MDA-MB-231 cells caused a reduction in SREBP-1 levels, which was more prominent in the precursor form. This effect was DGKζ-specific, since reduction of DGKα did not alter SREBP-1 processing, nor was it further affected by R59949 addition to DGKζ-depleted cells (**Figure 16C**). DGKζ overexpression increased SREBP-1 levels as well as its processing, an effect not

observed when a kinase-dead form of the enzyme was expressed (not shown). These data confirm that whereas both DGKα and DGKζ are able to regulate DAG effectors (the PKC-PKD axis), DGKζ has a critical metabolic role, as it is the main isoform involved in SREBP-1 control.

2.2. Contribution of the DGK pathway to the maintenance of the oncogenic axes that promote breast cancer progression

The previous experiments show that DGK pharmacological inhibition results in DAG accumulation that strongly affects lipid traffic and metabolism. DGK not only consume DAG, but also generate PA. We next evaluated if lack of PA production as a result of DGK inhibition had also some impact on breast cancer progression.

Diacylglycerol kinase inhibition abrogates Akt activation following rapamycin treatment

The MDA-MB-231 cell line is highly invasive and, like other breast cancer-derived cell lines, is rapamycin-resistant (Sarbassov et al., 2006). In these cells, high PA levels are suggested to reduce rapamycin sensitivity (Chen et al., 2003). We showed that DGK activity was severely reduced by 30 μM R59949 (**Figure 12A**). The effects of this dose were evaluated alone or in combination with rapamycin on mTOR substrates. Cell treatment with 100 nM rapamycin prevented mTORC1 activation, observed as a reduction in p70S6K phosphorylation and of its downstream substrate ribosomal protein S6 (rpS6; **Figure 17A**). Rapamycin inhibition of mTORC1 correlated with increased Akt phosphorylation at Ser473. R59949 alone had a minor effect on mTORC1 activity, but decreased Akt phosphorylation at Ser473. Together with rapamycin, the DGK inhibitor abrogated rapamycin-induced Akt phosphorylation. As a readout of Akt, we determined cyclin D3 levels. R59949 (24 h) decreased cyclin D3 expression, further indicating a correlation between DGK activity and Akt (**Figure 17A**).

In accordance with the effects of R59949 on S6K phosphorylation, short-term treatment with the inhibitor had little effect on mTORC1 assembly. Rapamycin nonetheless strongly reduced the interaction between raptor and mTOR (**Figure 17B**). Short-term (1 h) DGK inhibition together with rapamycin treatment decreased rictor association to mTOR only slightly. This effect was more pronounced after long-term

treatment (24 h); rapamycin dissociated 30% of the complex, whereas the use of both inhibitors reduced the amount of mTOR-associated rictor by nearly 80% (**Figure 17B**).

These results concur with the differences reported in rapamycin sensitivity shown by both mTOR complexes. The data also indicate that mTORC2 rapamycin sensitivity is clearly augmented by blocking a PA source, and that even when the mTOR/rictor complex is assembled, inhibition of DGK has a profound effect on Akt phosphorylation.

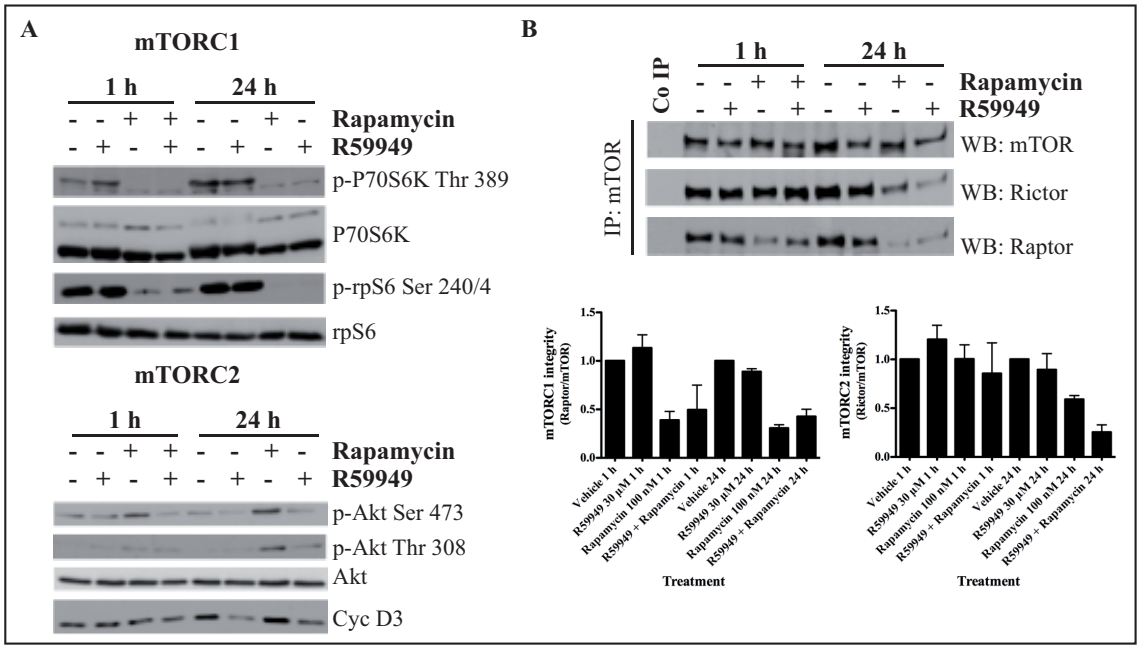


Figure 17. DGK inhibition blocks Akt phosphorylation and cooperates with rapamycin to disrupt mTORC2 in MDA-MB-231 cells. (A) To evaluate the DGK contribution to mTOR, cells were treated with R59949 (30 μM), rapamycin (100 nM), or both (1 or 24 h). Total cell lysates were analyzed by Western blot using the indicated antibodies. (B) Cells in exponential growth were treated as indicated (1 or 24 h) before lysis using CHAPS buffer. Protein (1 mg) in lysates was used to immunoprecipitate mTOR. Associated raptor and rictor were analyzed by Western blot. mTOR complex integrity was determined as the ratio of immunoprecipitated raptor/mTOR and rictor/mTOR. Mean ± SEM is shown for results from at least three independent experiments.

Diacylglycerol kinase inhibition delays G1-S cell cycle progression in MDA-MB-231 cells

The PI3K-Akt-mTOR axis plays a positive role in the cell cycle, facilitating cell cycle entrance and allowing the transition between the G1 and S phases. We examined the effect of R59949 and rapamycin treatment on mTOR activity throughout the cell cycle. MDA-MB-231 cells were synchronized by serum deprivation, and rpS6 and Akt phosphorylation evaluated after serum readdition. DGK inhibition decreased Akt phosphorylation, alone or in the presence of rapamycin **(Figure 18A)**. DGK inhibition markedly delayed cell cycle progression. At 12 h post-serum addition, nearly 65% of

vehicle-treated cells were in S phase and rapamycin-treated cells had entered S phase after a delay, whereas the majority of R59949-treated cells were in G1. DGK-inhibited cells resembled cells in a serum-starved state. The effect of co-treatment with rapamycin and R59949 was similar to that of R59949 (**Figure 18B, C**).

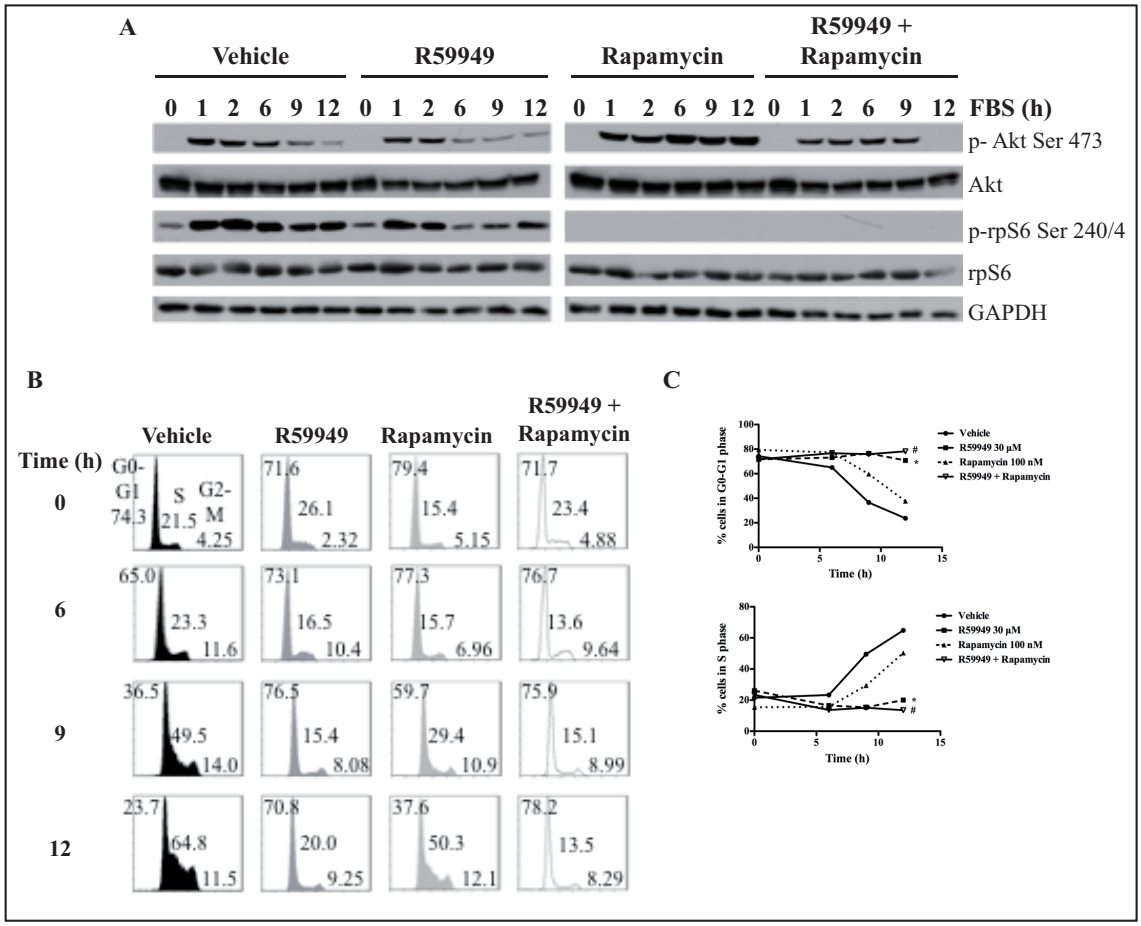


Figure 18. DGK inhibition delays Akt phosphorylation and G1-S progression in MDA-MB-231 cells. (A) Cells were serum-starved and cell cycle progression was followed after serum readdition. DMSO, R59949 (30 μM) and rapamycin (100 nM) were added 1 h before serum. Phosphorylation levels of S6 and Akt evaluated by Western blot. (B) Flow cytometry analysis using propidium iodide staining to determine the percentage of cells in each cell cycle stage. (C) The percentage of G0-G1 and S phase cells is indicated for the different treatments. * and # $p < 0.05$ compared to vehicle or rapamycin, respectively.

Diacylglycerol kinase inhibition promotes apoptosis of MDA-MB-231 cells

Akt has a positive role in proliferation, but also in survival and apoptosis prevention (Manning and Cantley, 2007); the effects of DGK inhibition on survival and apoptosis prevention were therefore determined. MDA-MB-231 cells were serum-starved and treated with different doses of the DGK inhibitor alone or together with rapamycin, and the apoptotic and death profile were determined using flow cytometry. Rapamycin reduced the percentage of apoptotic cells, whereas the DGK inhibitor greatly increased this percentage (**Figure 19A, B**). In agreement with the propidium iodide/annexin-V

data, DGK inhibition also increased PARP processing, another indicator of apoptosis (**Figure 19C**). These data thus indicate that DGK activity is necessary to prevent apoptosis and maintain the survival of MDA-MB-231 cells.

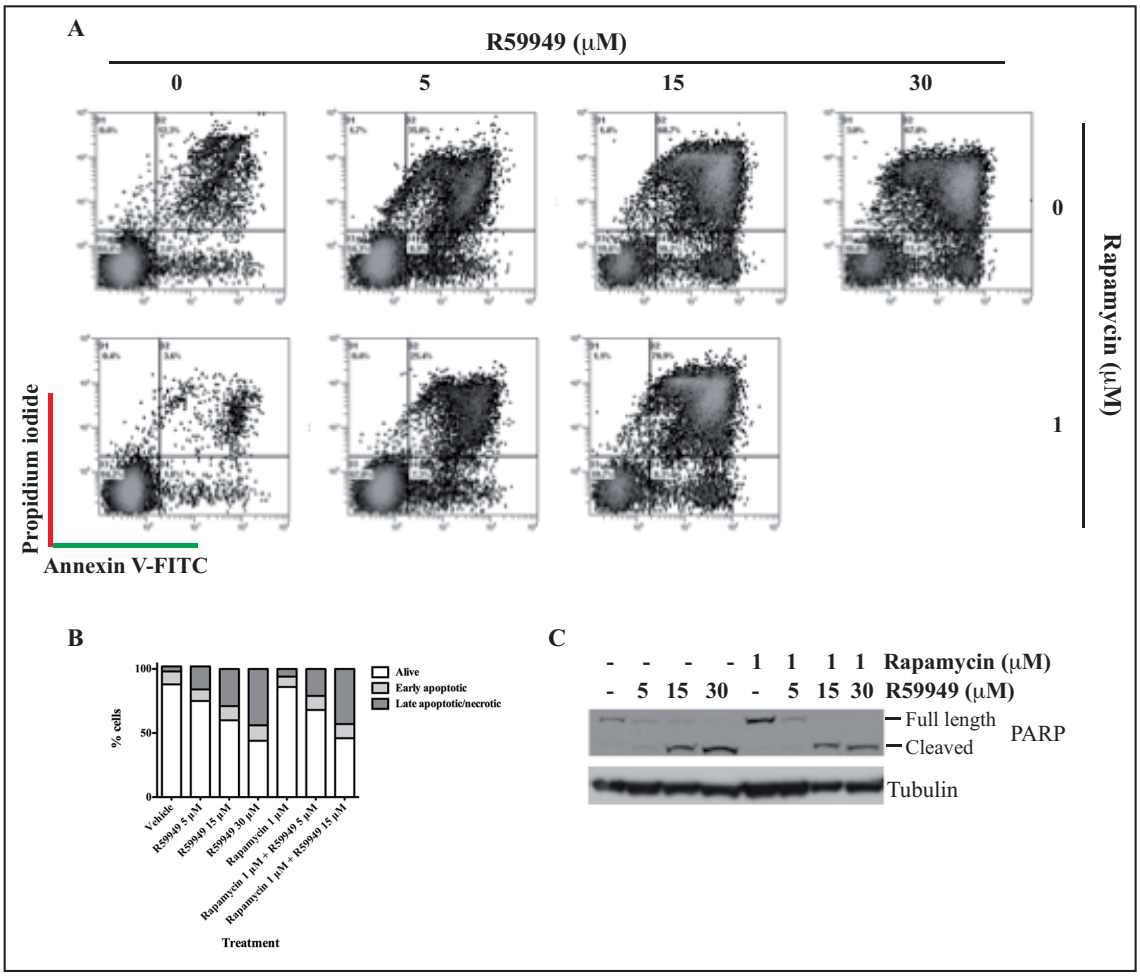


Figure 19. DGK activity prevents apoptosis of MDA-MB-231 cells. (A) MDA-MB-231 were serum-starved and treated (36 h) with R59949 or rapamycin at the indicated concentrations. Apoptosis was measured by flow cytometry. Data are from one representative experiment (n = 3). (B) Mean of live, early apoptotic (annexin V^+ propidium $iodide^-$) and late apoptotic/necrotic cells (annexin V^+ propidium $iodide^+$) from n = 3 experiments. (C) PARP cleavage was analyzed by Western blot of extracts from cells treated as in A).

The diacylglycerol kinase effect on Akt Ser473 phosphorylation is independent of mTOR/rictor association

Studies in mouse models have shown that mTORC2-Akt signaling is dependent on PI3K activity (Hietakangas and Cohen, 2007) (Chalhoub et al., 2009). PI3K activity is also important in rapamycin-elicited mechanisms, since this drug is proposed to derepress various negative feedback loops that limit PI3K-Akt activation (Harrington et al., 2005). We studied the effect of inhibiting DGK on mTOR complex formation and Akt phosphorylation in MCF-7 and MDA-MB-468 breast cancer cell lines. In contrast

to MDA-MB-231, these two cell lines are not reported to have high PA levels, but show hyperactivation of the PI3K pathway. MCF-7 cells have an oncogenic, activating mutation of the p110α PI3K catalytic subunit, whereas MDA-MB-468 cells do not express PTEN (Stemke-Hale et al., 2008).

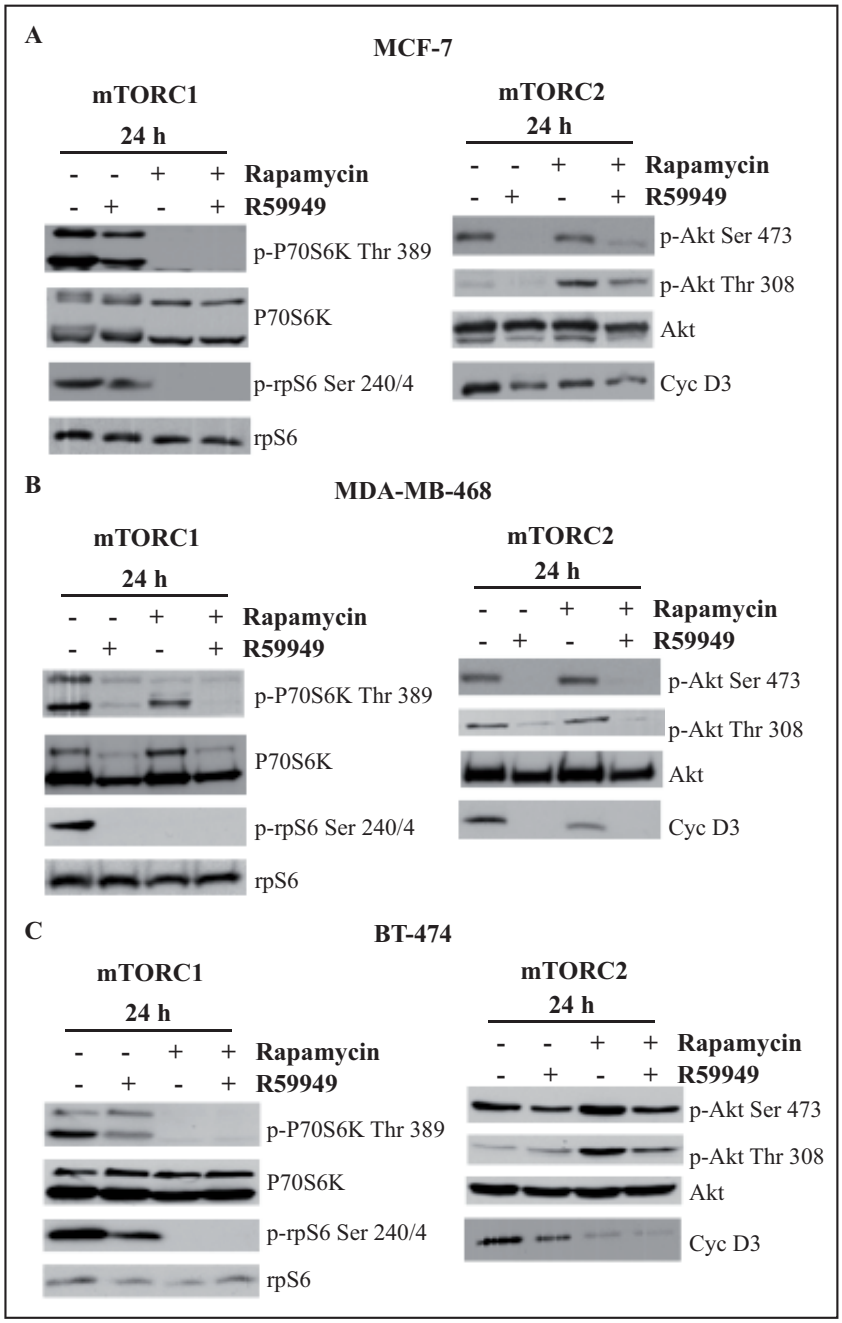


Figure 20. R59949 blocks Akt phosphorylation in breast cancer cells with hyperactive of PI3K signaling. (A-C) MCF-7, MDA-MB-468 and BT-474 cells in exponential growth were treated with DMSO, R59949 (30 μM), rapamycin (100 nM), or both (24 h). Total cell lysates were analyzed by Western blot using the indicated antibodies. One representative experiment is shown (n =3).

In these cell lines, rapamycin reduced S6K/rpS6 phosphorylation, but did not modify Akt phosphorylation; DGK inhibition decreased mTORC1 activity, although not

to the same extent as rapamycin. R59949 effects were small in MCF-7 cells, but pronounced in MDA-MB-468 cells. R59949, alone or in combination with rapamycin, greatly reduced Akt phosphorylation (**Figure 20A, B**). Results were similar in BT-474 cells, which overexpress the RTK HER2 (Erbb2, Neu; (Neve et al., 2006), and thus also show increased PI3K signaling (**Figure 20C**).

In contrast to MDA-MB-231 cells, mTOR complex assembly in MCF-7 and MDA-MB-468 cells was more sensitive to DGK inhibition (**Figure 21A, B**). In these cells, although rapamycin induced total disassembly of mTORC2, Akt phosphorylation was preserved.

These data indicated that, in cells with a hyperactivated PI3K pathway, Akt Ser473 phosphorylation can occur independently of mTOR/rictor assembly, supporting the existence of Akt Ser473 kinases in addition to mTORC2 (Troussard et al., 2006). In this case, mTORC2-independent Akt phosphorylation requires DGK activity. Although it contributes to mTOR complex assembly, DGK-derived PA thus has an additional, critical function as a positive modulator of the PI3K-Akt axis.

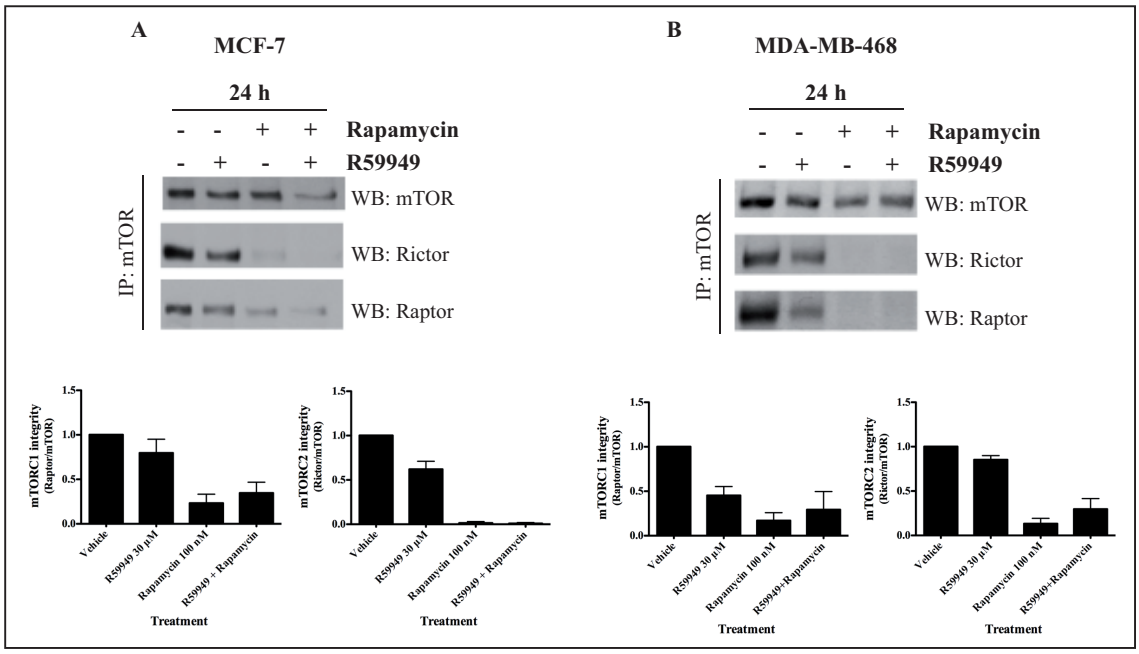


Figure 21. DGK inhibition modifies mTOR complex assembly in breast cancer cells with hyperactivation of PI3K signaling. (A, B) MCF-7 and MDA-MB-468 cells in exponential growth were treated with DMSO, R59949 (30 μM), rapamycin (100 nM), or both (24 h). Protein (1 mg) in CHAPS cell lysates was used to immunoprecipitate mTOR. Associated raptor and rictor were analyzed by western blotting. mTOR complex integrity was determined as the ratio of immunoprecitated raptor/mTOR and rictor/mTOR. Mean ± SEM is shown for results from at least three independent experiments.

DGK activity controls PI3K-Akt through generation of phosphoinositide intermediates and maintenance of phosphotyrosine signaling

Our data indicated that DGK activity is needed for adequate PI3K signaling; DGK might therefore be necessary to allow PI3K activation by facilitating its coupling to phosphotyrosine platforms. This is controlled by a balance between tyrosine and Ser/Thr phosphorylation of the receptors and scaffolds that dock PI3K through phosphotyrosine residues. In muscle cells, lack of DGK activity is associated with PKC activation, which prevents insulin receptor substrate 1 (IRS-1) coupling to PI3K in response to insulin (Chibalin et al., 2008). To determine whether the effect of DGK activity on Akt phosphorylation relies on the ability of DGK to modulate PKC signaling, we tested PKC inhibition on Akt Ser473 phosphorylation in MDA-MB-231 cells. PKC inhibition mimicked the effect of rapamycin on Akt phosphorylation; however, simultaneous inhibition of PKC and DGK activities abrogated Akt phosphorylation (**Figure 22A**). This suggests that DGK effects on Akt phosphorylation are not PKC-mediated, and that DGK and PKC operate in parallel loops.

We examined the profile of protein tyrosine phosphorylation in MDA-MB-231 cells treated with R59949, rapamycin or both. DGK inhibition induced a sharp decrease in protein tyrosine phosphorylation (**Figure 22B**). This finding suggested that DGK activity maintains a protein phosphotyrosine-mediated loop, which in turn might promote PI3K activity and Akt phosphorylation in response to rapamycin.

DGK could also promote PI3K activity by providing a critical PtdIns pool. The DGK are metabolic enzymes, and their essential function in PtdIns turnover is widely accepted (Jenkins et al., 1994) (Milne et al., 2008). We thus measured PtdIns-4-phosphate and $PtdInsP_2$ levels in MDA-MB-231 cells treated for 24 h with R59949. DGK inhibition decreased the levels of both lipids **(Figure 22C**), but did not modify the amount of radiolabeled PA (not shown). Treatment of the cells with LiCl, an inhibitor of inositol phosphatases and promoter of PA accumulation, reversed the effect of R59949 on Akt phosphorylation (**Figure 22D**). LiCl did not affect GSK3 phosphorylation (**Figure 22D**). Our data thus indicate that R59949 modulates a specific PA pool responsible of PI3K and mTORC2 activity.

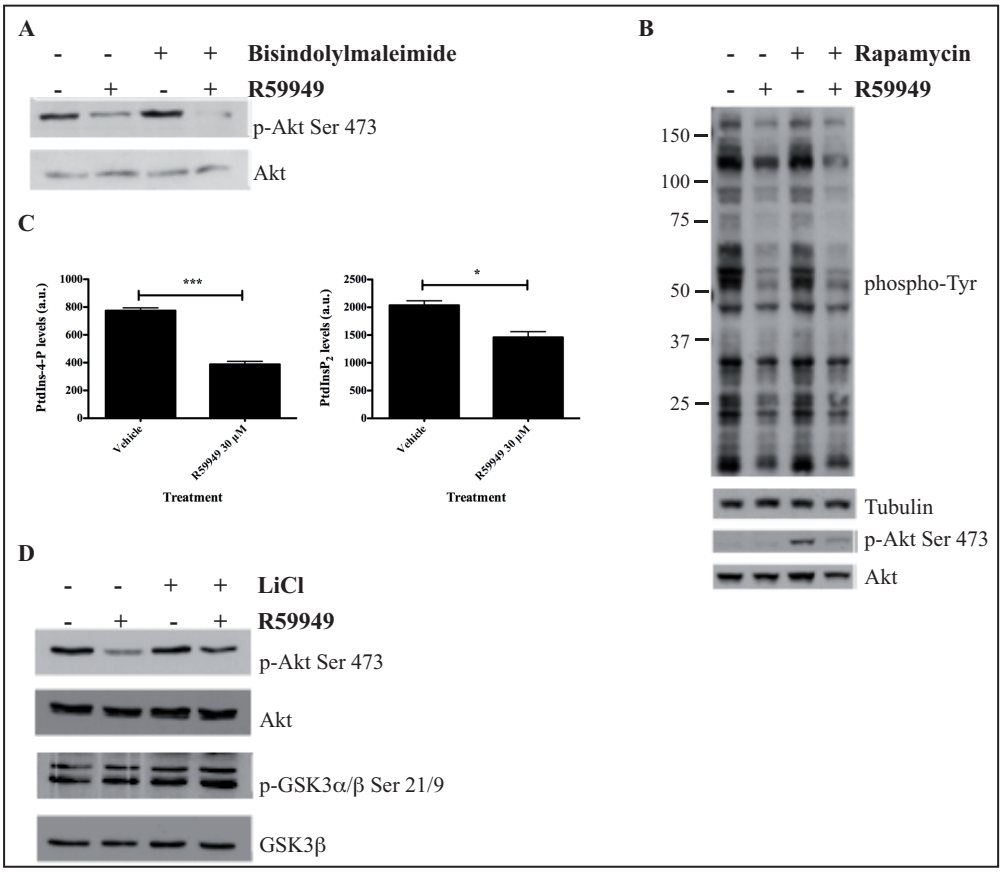


Figure 22. R59949 reduces PtdIns intermediates and protein tyrosine phosphorylation. (A) MDA-MB-231 cells in exponential growth were treated with DMSO or with R59949 (30 μM), the PKC inhibitor bisindolylmaleimide I (5 μM), or both (24 h). Total cell lysates were analyzed by Western blot with indicated antibodies. (B) The total protein phospho-Y profile was analyzed by Western blot in Triton-X100 lysates of MDA-MB-231 cells treated with R59949 (30 μM), rapamycin (100 nM), or both (24 h). Ser 473 phosphorylation and total Akt were evaluated as controls. (C) MDA-MB-231 cells were treated as indicated (24 h), followed by metabolic labeling (1 h) with ^{32}P-orthophosphate. Lipids were extracted and analyzed by TLC and HPLC. PtdIns-4-phosphate and $PtdInsP_2$ amounts are shown. Results are shown for a single experiment performed in triplicate, of three performed with similar results. (D) Cells were seeded and treated as in A) but with LiCl (0.5 mM). *p <0.05, ***p <0.001.

DGKα and DGKζ control mTORC1 activity, but only DGKα is required for Akt and Src activation

The previous results indicated that DGK activity is essential for correct mTORC1 and mTORC2 activation in breast cancer-derived cell lines. To identify the isoform responsible for this effect, we used MDA-MB-231 cells depleted of DGKα or DGKζ. We first evaluated P70S6K activation following mitogenic stimulation. A reduction in any DGK isoform abrogated P70S6K activation, measured as Thr389 phosphorylation at early times after serum addition (2 h; **Figure 23A**). Nonetheless, the second peak of P70S6K activation (from 15 to 18 h post-stimulation) was only affected by DGKζ depletion (**Figure 23A**). These data indicate that although both isoforms are necessary

for early mTORC1 activation, DGKζ is the main contributor to the activity of this complex, as it also affects the late activity peak.

DGK also promoted Akt Ser473 phosphorylation. In this case, the DGKζ reduction provoked a sharp decrease in Akt phosphorylation at this residue, but only depletion of DGKα completely abrogated it. When we treated the cells with rapamycin, however, none of the DGK isoforms were able to revert rapamycin-induced Akt Ser473, at difference to findings for the DGK inhibitor R59949 (**Figure 23B**). We observed an ~90% reduction of protein levels with RNAi; rapamycin might therefore activate the remaining DGK or another isoform to promote Akt phosphorylation. Akt activation requires $PtdInsP_3$ generation by the PI3K, whose activity depends on tyrosine signaling. We analyzed activation of SFK, a family of non-RTK responsible for transducing and maintaining phosphotyrosine signaling. Consistent with our previous observation, depletion of DGKα impaired Src activation (**Figure 23C**). Our data therefore place DGKα as an essential regulator of Akt and SFK.

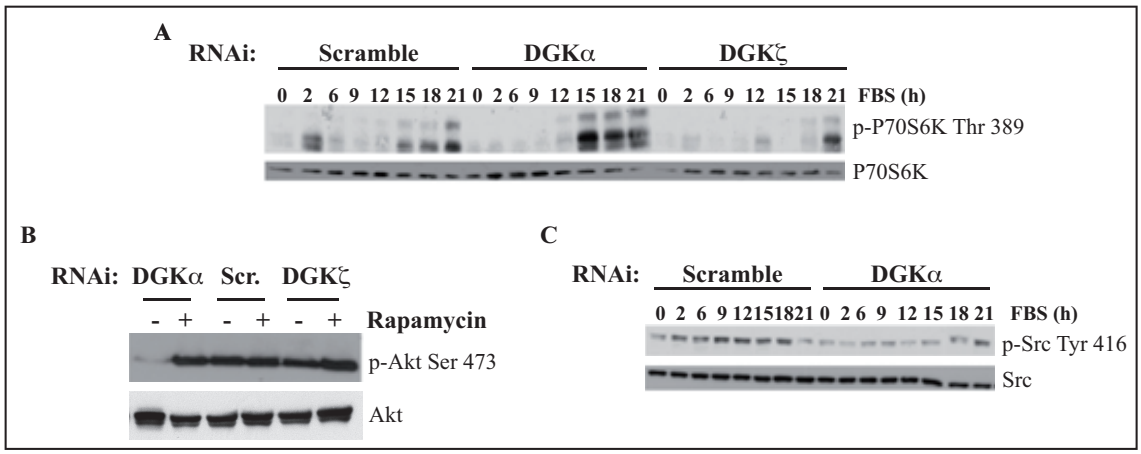


Figure 23. Depletion of DGKα and DGKζ impairs P70S6K phosphorylation, but only DGKα is necessary for Akt and Src activation. (A) DGKα or DGKζ levels were reduced in MDA-MB-231 cells. Cells were serum-starved (24 h) and allowed to enter the cell cycle after serum readdition. P70S6K kinase phosphorylation was evaluated by Western blot. (B) DGKα or DGKζ levels were reduced in MDA-MB-231 cells. Cells were treated (24 h) with DMSO or rapamycin (100 nM). Akt phosphorylation was evaluated by Western blot. (C) Cells as in A). Src activation was analyzed by Western blot to detect tyrosine 416 phosphorylation. Results are shown for one representative experiment (n =3).

2.3. Contribution of DGK to the development of pharmacological resistance in breast cancer cells

Our observations indicated that, by consuming DAG or generating PA, the DGK pathway makes a positive contribution to oncogenic axes responsible for breast cancer maintenance. DGK activation could therefore be an intermediate in pharmacological

resistance pathways. In addition, inhibition of DGK could promote the switching on of new pathways that could render the cell resistant. We therefore evaluated whether DGK activity was activated as a result of pharmacological intervention, and if DGK inhibition promoted the activation of other oncogenic pathways.

DGK is a component of the rapamycin-induced feedback loop that controls PI3K-Akt signaling

Our findings in MDA-MB-231 cells indicated that DGK activity participates in the rapamycin-induced feedback loops, and that DGK activity is required for rapamycin-induced Akt phosphorylation. To better understand the molecular mechanism by which DGK modulates rapamycin responses, we evaluated DGK activity in membrane fractions of MDA-MB-231 cells after several pharmacological treatments. As predicted, R59949 treatment inhibited DGK activity in this fraction. Rapamycin treatment induced a sharp increase in DGK activity, which was prevented by R59949 (**Figure 24A, top**). We used an *in vitro* assay to assess the contribution of DGK activity to DAG levels by transforming endogenous DAG into PA. In accordance with the data for endogenous DGK activity, DAG levels increased following DGK inhibition and decreased after rapamycin treatment (**Figure 24B**).

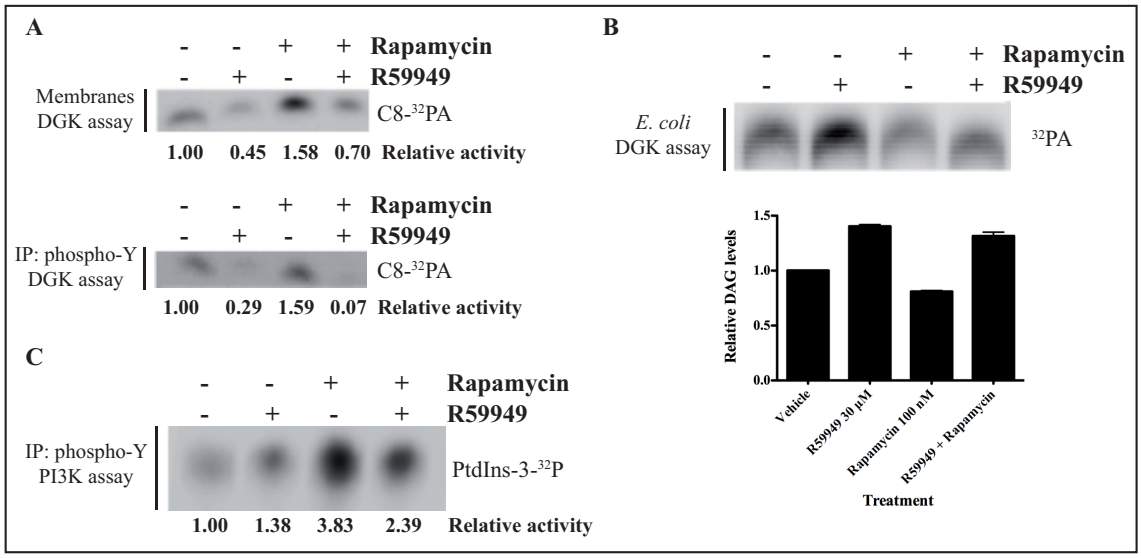


Figure 24. DGK activity is induced by rapamycin treatment to promote PI3K signaling. (A) MDA-MB-231 cells in exponential growth were treated (24 h) as indicated. DGK activity was determined in membranes (top) or in phosphotyrosine immunoprecipitates (bottom). Relative DGK activity is indicated beneath autoradiograms (DGK activity of vehicle-treated cells = 1.00). (B) Total cell DAG was determined in an *in vitro* radioenzyme assay. A representative autoradiogram and the mean ± SEM of at least three independent experiments are shown. (C) PI3K activity was evaluated in phosphotyrosine immunoprecipitates. Relative PI3K activity is indicated beneath the autoradiogram (PI3K activity of vehicle-treated cells = 1.00).

One of the best-characterized feedback loops derepressed by rapamycin is the Ser/Thr phosphorylation of receptors and scaffolds responsible for tyrosine phosphorylation-mediated tethering of PI3K regulatory subunits to signaling complexes (Harrington et al., 2005). Rapamycin treatment led to an increase in phosphotyrosine-associated PI3K activity (almost four times control values). The increase was reversed when DGK was inhibited (**Figure 24C**). We also detected DGK activity in phosphotyrosine immunoprecipitates in both control and rapamycin-treated cells, but not following R59949 treatment (**Figure 24A, bottom**).

These results led us to conclude that DGK activity parallels rapamycin-enhanced PI3K activation, suggesting that rapamycin triggers the generation of signaling lipids through activation of enzymes such as PI3K and DGK. Our data also revealed that enhanced PI3K coupling to phosphotyrosine requires DGK activity.

DGK inhibition does not reverse feedback mechanisms that rely on RTK-ERK activation

Some tumors are refractory to current treatments, a resistance that is normally acquired from the unexpected activation of feedback loops elicited by the drugs themselves. mTORC1 inhibition with rapamycin promotes not only PI3K-Akt activation, but also ERK activity as a result of derepression of mTOR-controlled feedback (Carracedo et al., 2008). Inhibition of Akt is reported to increase RTK-mediated signaling and ERK hyperactivation (Serra et al., 2011) (Chandarlapaty et al., 2011). At least in T cells, DGK inhibition promotes Ras-dependent ERK activation (Sanjuan et al., 2001). As DGK inhibition inactivates the PI3K-Akt-mTOR axis, we tested whether it could switch on tyrosine and ERK signaling in breast cancer-derived cell lines, since this might promote resistance to a DGK-based pharmacological therapy. Treatment of breast cancer-derived cell lines with R59949 reduced total phoshphotyrosine signaling in all cell lines tested (**Figure 25A**).

We next evaluated how DGK inhibition, alone or with rapamycin, affected ERK activation. As expected, rapamycin maintained or increased ERK phosphorylation. Inhibition of DGK prevented ERK activation, since less ERK phosphorylation was observed, and reversed rapamycin effects (**Figure 25B**). These data suggest that DGK activity is needed to sustain phosphotyrosine activity and that, in breast cancer cell lines, ERK activation might depend on DGK-produced PA.

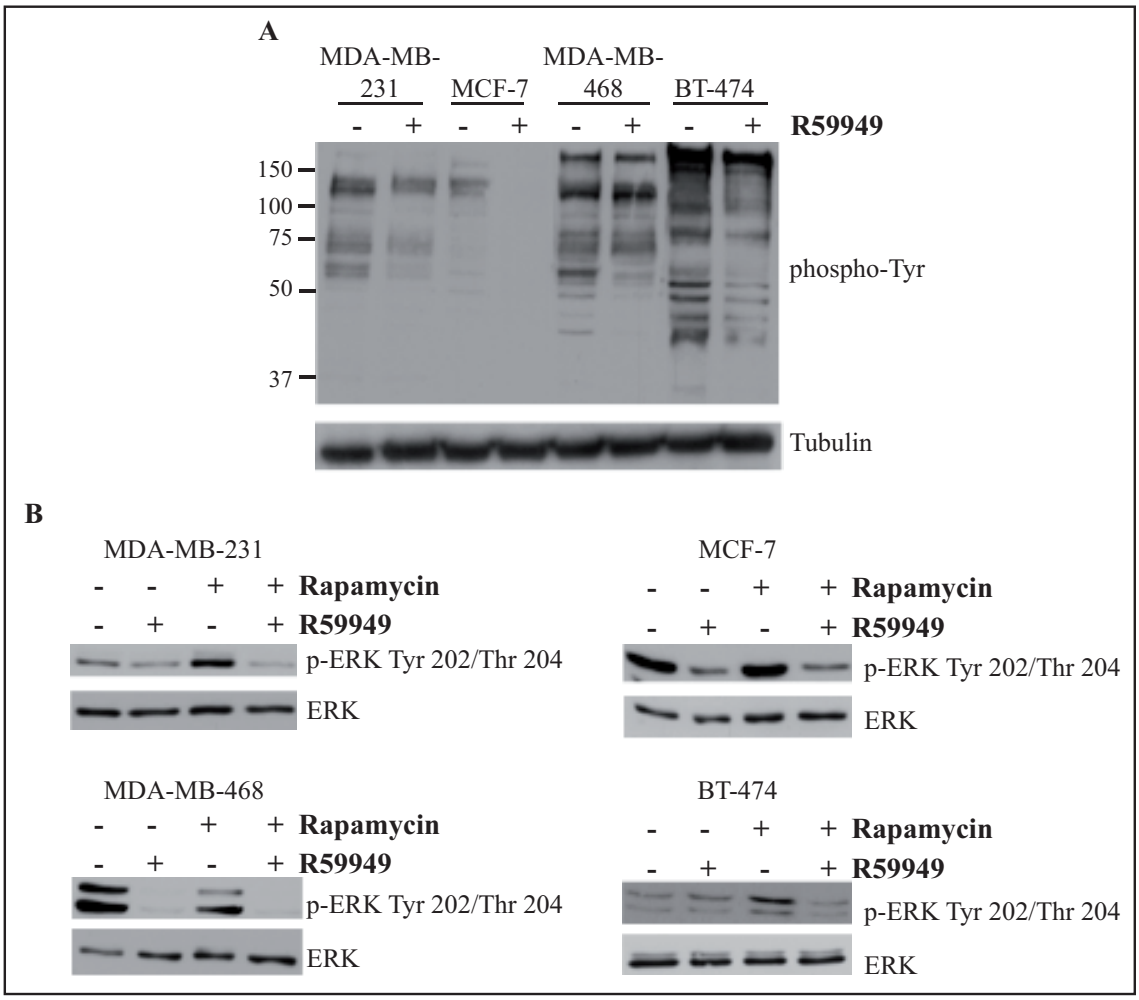


Figure 25. DGK inhibition prevents tyrosine and ERK signaling in breast cancer cells. (A) MDA-MB-231, MCF-7, MDA-MB-468 and BT-474 cells in exponential growth were treated with R59949 (30 μM; 24 h). The phosphotyrosine profile was examined by Western blot. (B) As in A), cells were treated with R59949 (30 μM) and rapamycin (100 nM). ERK activation was evaluated by Western blot. Data are from one representative experiment (n =3).

DGKα is necessary for HIF-1α accumulation in MDA-MB-231 cells

The hypoxia-inducible factor (HIF)-1α is a transcription factor whose accumulation is promoted by mTOR activation. Accumulation of HIF-1α activates a transcription program that allows tumor cell adaptation to hypoxic environments and provides survival advantages, including resistance to chemo- and radiotherapy (Harris, 2002). As we observed that the DGK are central regulators of mTOR, we analyzed how the DGK affect HIF-1α. We assessed the effect of inhibiting DGK on mitogen-induced HIF-1α accumulation. Treatment of cells with R59949 abrogated HIF-1α accumulation in the MDA-MB-231 cell line after serum addition (**Figure 26A**). We performed the same kind of experiments in cells transfected with RNAi to DGKα or DGKζ. DGKζ depletion promoted a slight reduction in HIF-1α levels, which were clearly abrogated

by depletion of DGKα (**Figure 26B**). These data suggest that DGKα is essential for HIF-1α accumulation in tumor cells, and confirm the relation between this isoform and mTOR activation.

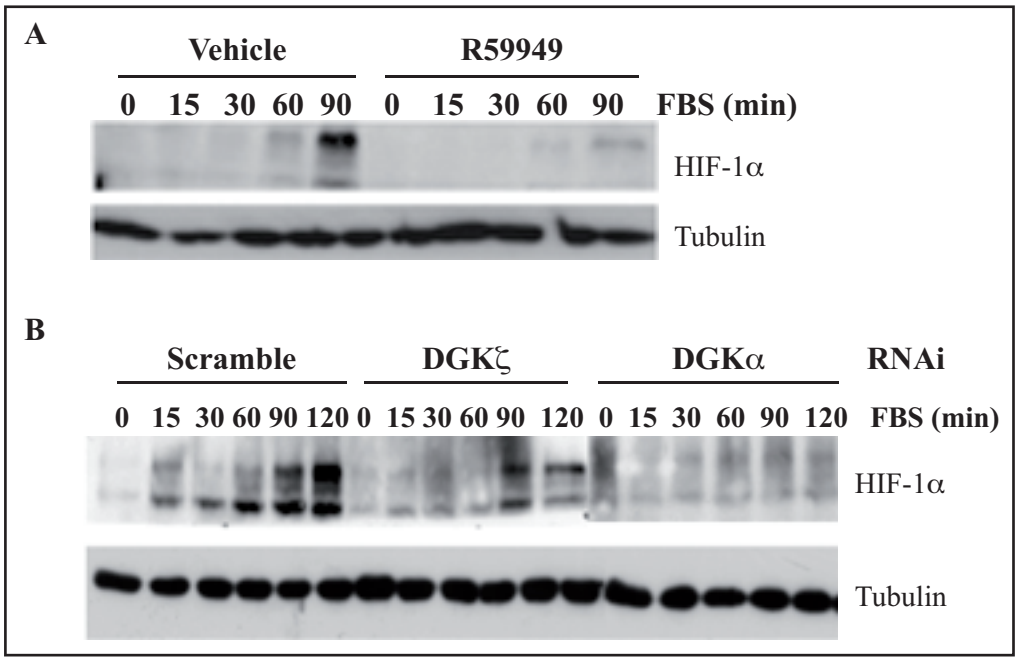


Figure 26. DGKα is necessary for HIF-1α accumulation after mitogenic stimulation. (A) MDA-MB-231 cells were serum -tarved (24 h) and treated with DMSO or R59949 (30 μM; 30 min). Serum was added for the indicated times and HIF-1α levels analyzed by Western blot. (B) MDA-MB-231 cells were depleted of DGKα or DGKζ. Cells were serum-starved (24 h), and stimulated for the indicated times. HIF-1α accumulation was evaluated by Western blot. Results are from one representative experiment (*n* =3).

3. RELEVANCE OF DGK EXPRESSION AND/OR ACTIVITY FOR BREAST CANCER INITIATION AND PROGRESSION *IN VIVO*

Our results indicated that DGK activity is central to the control of DAG levels to maintain adequate lipid metabolism of tumor cells. In addition, DGK operates through several interconnected mechanisms, to maintain the supply of PtdIns that drives the PI3K-Akt-mTOR axis in breast cancer cells. Targeting DGK could thus impair/reduce tumor growth, alone or in combination with other treatments.

DGK inhibition impairs breast cancer cell proliferation *in vitro*

To study the potential for pharmacological intervention in this pathway, we assessed the effect of DGK inhibition on long-term growth of MDA-MB-231 cells in colony formation assays of cells cultured for several days with various doses of R59949. Inhibitor treatment led to a reduction in colony number, and 20 μM R59949 resulted in

~50% reduction in cell growth (**Figure 27A**). Short-term rapamycin treatment did not alter the growth of these cells, but long-term treatment reduced colony numbers. This observation concurs with a report showing the effects of rapamycin on long-term growth of MDA-MB-231 cells (Breuleux et al., 2009). Combined treatment with both drugs slightly reduced cell growth when <20 μM DGK inhibitor was used, indicating an additive effect (**Figure 27A, C**).

The effects of R59949 on colony formation were also evaluated in the cell lines with a hyperactivated PI3K pathway. In these cells, DGK inhibition markedly reduced colony number (**Figure 27B, C**). R59949 sensitivity was inferred by comparing the IC_{50} for each cell line. MDA-MB-231 cells showed the highest IC_{50} (22 μM), whereas cells with increased PI3K activity had a lower IC_{50} (12 to 18 μM; **Figure 27D**). These variations in sensitivity to DGK inhibition support a relationship between DGK activity and aberrant PI3K activation, as well as the differences in sensitivity of both complexes to rapamycin and DGK inhibitor.

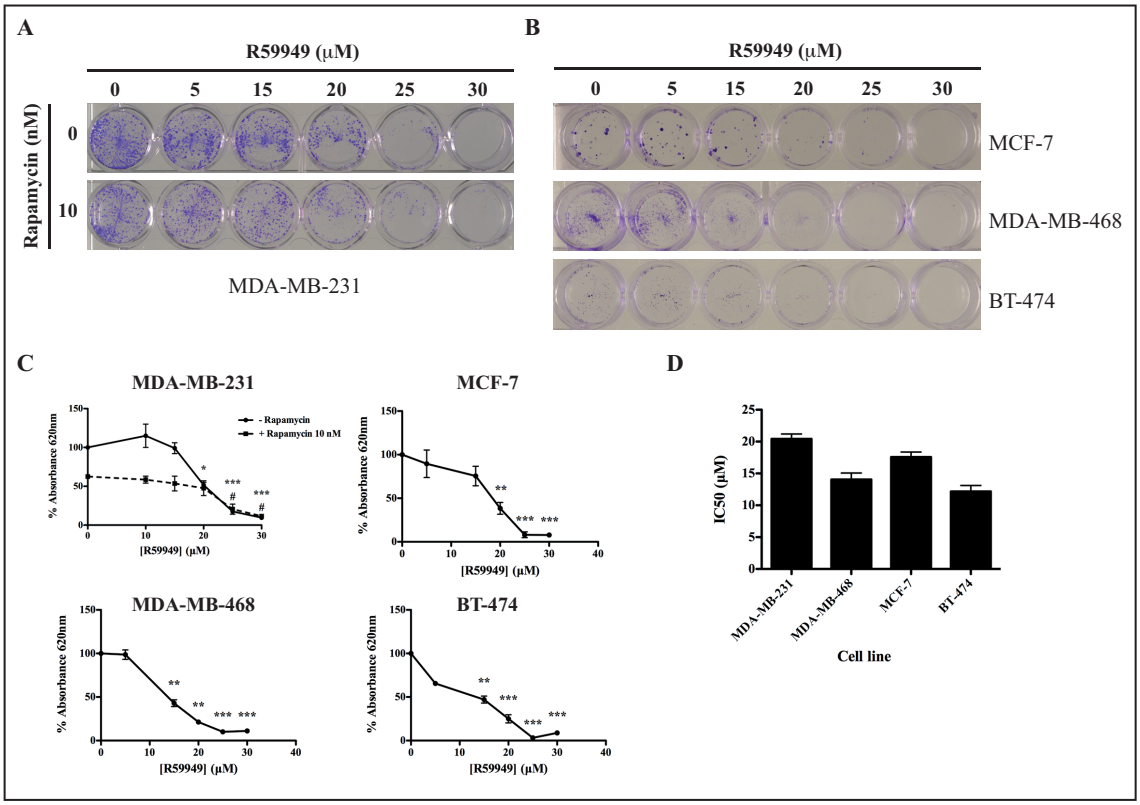


Figure 27. DGK inhibition impairs cell proliferation in breast cancer-derived cell lines. (A) Colony growth formation assays were performed using MDA-MB-231 cells treated (10 days) with DMSO, R59949 and/or rapamycin (10 nM). (B) Colony growth formation assays were evaluated in MCF-7, MDA-MB-468 and BT-474 cells treated (10 days) with DMSO or R59949. (C) Crystal violet staining was quantified to evaluate colony number and size. (D) IC_{50} of R59949 was determined for each cell line. Data show mean ± SEM for triplicate samples from one representative experiment ($n \geq 3$; triplicates for each condition). Student's *t*-test, *p <0.05; ** <0.01; *** <0.001.

Diacylglycerol kinase ζ is necessary to maintain proliferation of breast cancer cells *in vitro*

Using the same assay, we tested the effect of DGKα or DGKζ reduction. DGKα depletion did not affect MDA-MB-231 cell growth, whereas DGKζ depletion significantly compromised cell growth *in vitro*, which was reduced by ~50% (**Figure 28**). This finding confirms the central role of DGKζ in the control of metabolism and promotion of tumor growth.

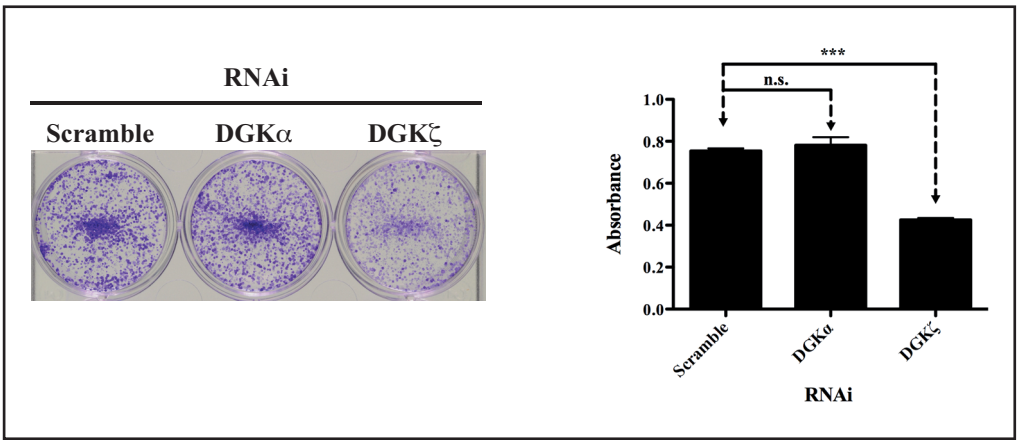


Figure 28. DGKζ is necessary for breast cancer cell colony growth. Colony growth formation assays were performed using MDA-MB-231 cells with stably reduced DGKα or DGKζ levels; crystal violet staining (left) and quantification of absorbance at 620 nm (right). The data show a representative experiment ($n \geq 3$; triplicates for each condition). Student's *t*-test, ***p <0.001.

Diacylglycerol kinase inhibition impairs breast tumor growth *in vivo*

The previous experiments suggested that DGK inhibition could impair or reduce *in vivo* tumor growth. We tested this by assessing the effect of R59949 in a preclinical study of heterotopic tumor generation in immunocompromised (SCID) mice; we used the MDA-MB-231 cell line to produce tumors, since this line is highly aggressive and had the highest IC_{50} to the drug, although it remained R59949-sensitive. When tumors reached 150 mm^3, mice were randomly separated into two groups, and we established an R59949 administration schedule using the maximum inhibitor dose that showed no toxic effects by subcutaneous delivery. Administration of R59949 (10 mg/kg/treatment) severely inhibited tumor growth (**Figure 29A**). Akt Ser473 phosphorylation and cleaved caspase 3 were evaluated by immunohistochemistry (IHC) in tumors. R59949 treatment decreased Akt Ser473 phosphorylation signal, and markedly increased the cleaved caspase staining (**Figure 29B**).

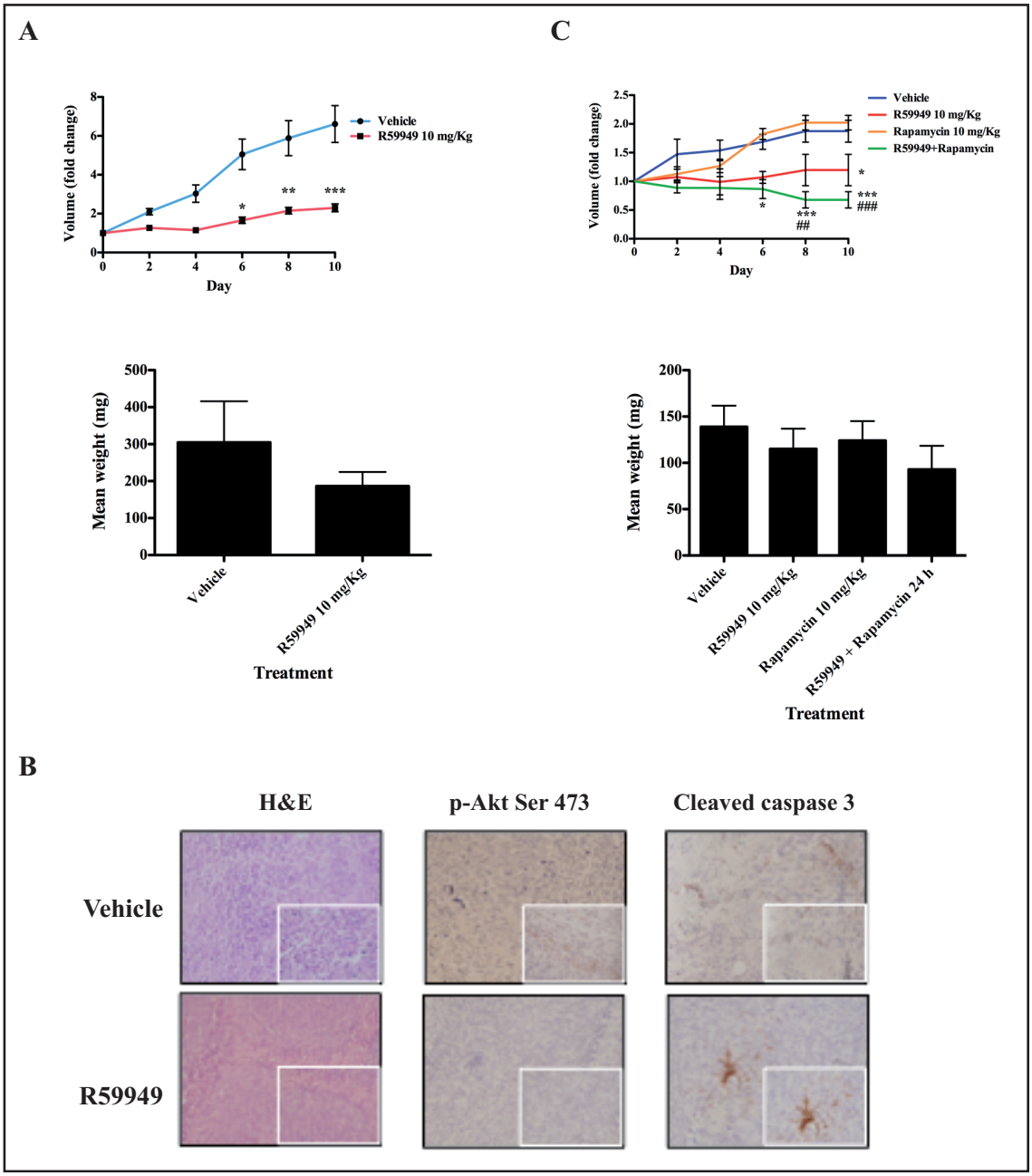


Figure 29. DGK inhibition impairs growth of MDA-MB-231-induced tumors. (A) 1.0 x 10^6 cells were injected into immunocompromised mice. When tumors reached ~150 mm^3, vehicle or R59949 (10 mg/Kg/48 h) were administered s.c. for several days. Tumor volume was recorded every third day to obtain a growth curve (left). At the end of the treatment, mice were sacrificed, tumors extracted and weighed (right). (B) Tumor sections were immunostained for phosphorylated Akt (Ser473) or cleaved caspase 3, together with hematoxylin. A magnification is shown in the inset. (C) As in A), except that 1.5 x 10^6 cells were injected into mice and rapamycin (10 mg/Kg/48 h) was administered alone or with R59949. *p <0.05; **<0.01; *** <0.001 compared to vehicle. ##p <0.01; ### <0.001 compared to rapamycin. The data show mean ± SEM for a representative experiment of at least three performed (*n* = at least 6 mice/group).

In another set of experiments, we administered rapamycin alone or with R59949. Rapamycin delayed the tumor growth rate only at the outset of treatment. The DGK inhibitor again reduced tumor growth, and showed a greater effect when combined with

rapamycin (**Figure 29C**). These data indicate that, in addition to reducing tumor progression on its own, R59949 could be applied in combination with rapamycin to treat tumors with characteristics similar to those of MDA-MB-231 cells.

Diacylglycerol kinase α is essential to sustain breast tumor growth *in vivo*

The previous experiments suggested that DGKζ has a major metabolic and signaling role, and that its inhibition reduced *in vitro* tumor growth. DGKα had minor effects, but was a critical regulator of tyrosine phosphorylation. We assessed the effect of depleting DGKα and DGKζ in MDA-MB-231 cells in a mouse xenograft model. DGKζ-depleted cells grew more slowly *in vivo* than control MDA-MB-231 cells, measured as the *x*-fold change in tumor volume. Cells lacking DGKα initially grew at the same rate as control cells, but tumors finally stopped growing and began to decrease in size (**Figure 30A**). When tumors were extracted and weighed, however, only those originating from DGKα-depleted cells were significantly smaller (approximately 50%; **Figure 30B**).

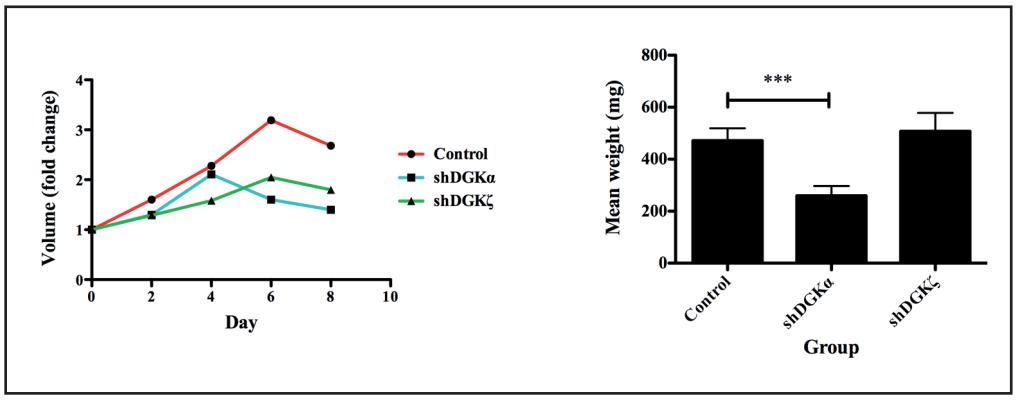


Figure 30. DGKα is necessary for breast tumor growth *in vivo*. 1.0 x 10^6 MDA-MB-231-shRNA-control, -shRNA-DGKα or -shRNA-DGKζ cells were injected into immunocompromised mice. Tumor volume (when ~150 mm^3) was recorded every two days for two weeks to obtain a growth curve (left). At the end of the treatment, mice were sacrificed, tumors extracted and weighed (right). ***p <0.001. The data show mean or mean ± SEM for a representative experiment of at least three performed (*n* = at least 6 mice/group).

Diacylglycerol kinase α is needed for MDA-MB-231 cells growth in 3D culture

These data suggest that DGKα is necessary for the *in vivo* growth of MDA-MB-231-derived tumors, although it has a modest effect on cell growth *in vitro*. New evidence suggests that the expression and function of many genes differs in epithelial cells, depending on whether they are cultured in 2D or in 3D matrices that mimic the ECM structure (Kenny et al., 2007). We thus tested whether this was the case for

DGKα. As a first approach, we assessed whether DGKα activity or levels depended on cell contact. Seeding of MDA-MB-231 cells at various confluences indicated that DGK activity and DGKα protein levels increased as the culture became more confluent (**Figure 31A**).

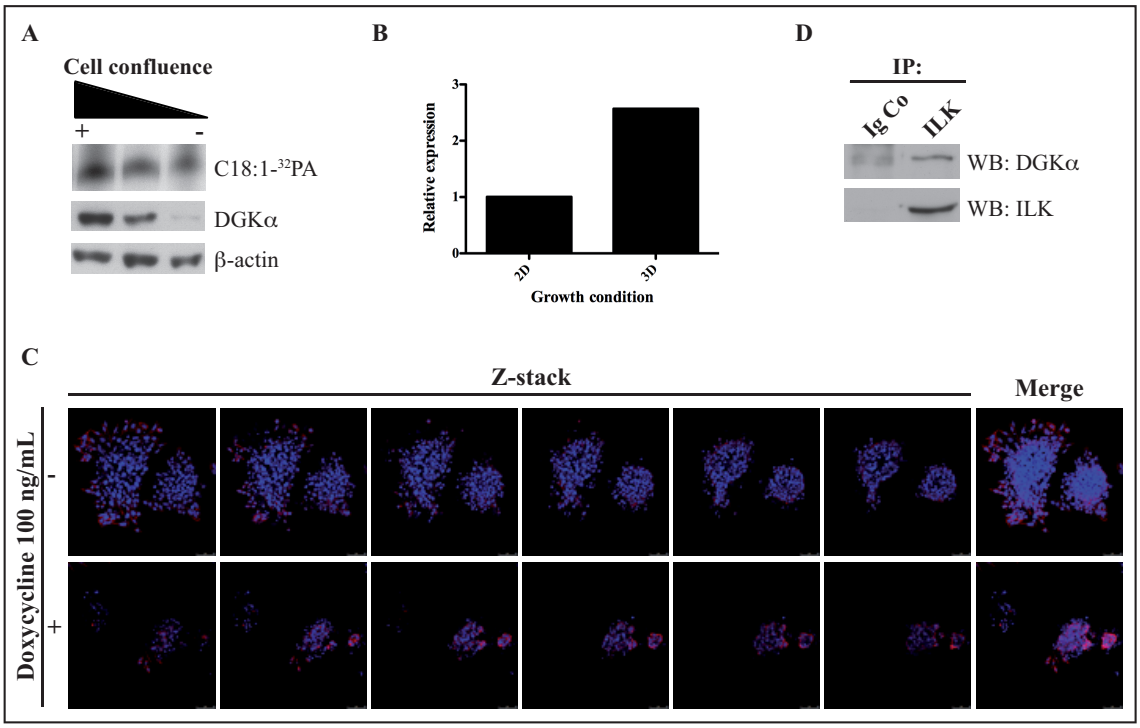


Figure 31. DGKα is necessary for MDA-MB-231 cell growth in 3D culture. (A) MDA-MB-231 cells were seeded at different densities. DGK activity was measured in whole cell extracts and DGKα analyzed by Western blot. (B) MDA-MB-231 cells were seeded on top of matrigel (4 days). RNA was extracted and *DGKα* mRNA expression analyzed by qPCR. (C) MDA-MB-231-pLKO-TetON-shDGKα cells were embedded in matrigel and allowed to grow for 12 days, alone or with doxycycline (100 ng/mL). Cells were stained with DAPI (blue) and rhodamine-phalloidin (red). (D) ILK was immunoprecipitated and DGKα detected by Western blot. Data are representative of one experiment (n = 3).

We next analyzed whether DGKα expression changed in 2D compared to 3D culture. We established an "on-top" matrigel culture and compared DGKα expression by qPCR. DGKα mRNA levels increased by nearly 3-fold when grown on ECM (**Figure 31B**). This increase suggested that DGKα could be needed to establish and maintain correct tissue architecture. We thus monitored the effect of DGKα deficiency on the growth of matrigel-embedded cells. We established stable MDA-MB-231 cells that could be DGKα-depleted in a doxycycline-dependent manner. When DGKα was reduced, MDA-MB-231 formed fewer and smaller groups than control cells (no doxycycline addition). DAPI staining of DGKα-depleted MDA-MB-231 cells showed that this could be a result of increased cell death, as more bubbling-staining was

observed (**Figure 31C**). Moreover, we found that DGKα associated to the integrin-linked kinase (ILK), a protein involved in the transduction of signals from the ECM (**Figure 31D**). These data could help to explain why DGKα depletion impaired *in vivo* tumor growth. Further studies are nevertheless required to establish a causal relation between DGKα and the 3D growth of tumors.

4. A REFERENCE FRAMEWORK TO EVALUATE THE POTENTIAL OF THE DGK PATHWAY AS A TARGET FOR THERAPEUTIC INTERVENTION

Our data indicated that DGKα and DGKζ play a positive role in breast cancer progression. DGKζ controls DAG levels to maintain tumor lipid metabolism and promotes tumor proliferation *in vitro*. DGKα nonetheless appears to be critical for tumor growth and maintenance *in vivo*. Inhibition of DGK proved effective in impairing tumor development. We also found that the DGK pathway can be a component of loops that promote resistance to tumor therapies. DGK targeting could thus be a promising approach for the treatment of breast cancer. As it is necessary to determine how DGKα and DGKζ levels are affected in patient samples, we first determined the relative expression of both isoforms in normal human breast, using formalin-fixed paraffin-embedded (FFPE) sections. qPCR analysis showed that DGKα was the most expressed isoform, with expression 34 times higher than that of DGKζ (**Figure 32A**).

We next compared the expression of the two isoforms in normal and malignant tissue. DGKα levels were increased in tumor samples, which did not include any samples of the $HER2^+$ subtype (**Figure 32B**). We analyzed DGKα levels in two $HER2^+$ breast tumors and found that DGKα expression was reduced by almost 80% compared to normal tissue (not shown). There were no differences in DGKζ levels between normal and malignant tissue (**Figure 32C**). Although these data are preliminary, combined with our previous observations, these findings allow us to hypothesize that certain DGKα levels might drive breast cancer progression, and could be effectively targeted for the treatment of breast tumors.

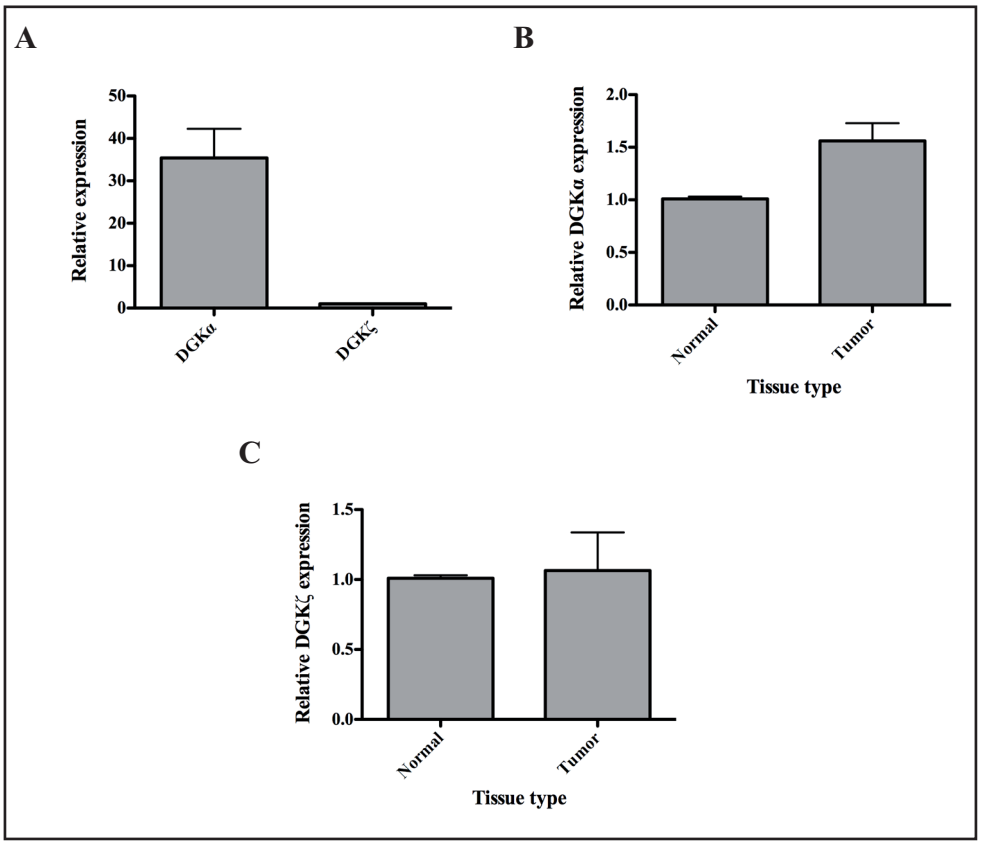


Figure 32. DGKα and DGKζ expression in human normal and tumor-derived breast tissue. DGKα and DGKζ expression was evaluated by qPCR in FFPE tissue sections. (A) Relative expression of DGKα and DGKζ in the normal breast (n = 5 samples). Relative expression of DGKζ was used as control. (B) Relative expression of DGKα in normal (n = 5) and tumor-derived tissue (n = 9). (C) Relative expression of DGKζ in normal (n = 5) and tumor-derived tissue (n = 9).

DISCUSSION

1. Strong DGKα expression in untransformed mammary cells suggests a tumor suppressor role

The DGK are a large family of lipid kinases whose functions have been broadly characterized in the immune and central nervous systems. At present, the only data that suggest a role for DGK in cancer are derived from microarray analyses; they mainly implicate the DGKα isoform, and there are no extensive studies on the contribution of specific DGK isoforms to the malignant phenotype.

In our study, we determined the expression pattern of the DGK isoforms in normal and breast cancer-derived cell lines. RT-PCR analysis of DGK expression in five selected cell lines showed that each cell line expressed at least five DGK isoforms. We then focused on the study of DGKα and DGKζ. Several studies implicate DGKα in tumor progression, but its function is debated, as it might have roles both as a tumor promoter and a tumor suppressor (Merida et al., 2009). At least in the immune system, DGKζ is highly redundant with DGKα, and mice deficient in either isoform share a phenotype with T cell hyperresponsiveness and resistance to anergy (Olenchock et al., 2006; Zhong et al., 2003). In our study, DGKα protein levels were severely reduced in all tumor-derived cell lines, whereas those of DGKζ tended to be higher in breast cancer cell lines. The reduction in DGKα was more prominent in those cell lines bearing mutations that lead to aberrant activation of the PI3K-Akt axis. We show that *DGKα* mRNA levels as analyzed by qPCR are significantly reduced in these cell lines compared to the untransformed MCF-10A cell line, and that they correlate inversely with the degree of phosphorylation of FoxO transcription factors. Hyperactivation of the PI3K-Akt axis leads to constitutive phosphorylation of FoxO, causing its inactivation. Our laboratory described three putative FoxO-binding sites in the *DGKα* gene promoter, which explains why DGKα is reduced in this subgroup of breast cancer-derived cell lines. This regulation appears to be DGKα-specific, as other isoforms such as DGKζ, are not regulated by FoxO- or PI3K-Akt-dependent mechanisms (Martínez-Moreno et al., under revision).

We tested whether DGKα expression correlated with DGK activity in our cell panel, and found that total DGK activity was higher in the untransformed cell line MCF-10A; the differences observed among the cell lines can be explained in terms of differential DGKα expression.

The characterization of FoxO-mediated regulation of *DGKα* throws new light on the conflicting data regarding the contribution of DGKα to malignant transformation. Studies of lymphoma and epithelial cells indicate that DGKα activity is coupled to tyrosine signaling (Bacchiocchi et al., 2005; Baldanzi et al., 2004; Cutrupi et al., 2000), and that DGKα-mediated PA generation promotes Rac and PKCζ activation (Chianale et al., 2010); the authors thus propose a positive DGKα contribution to tumor survival, angiogenesis and/or migration. Conversely, DGKα is reported to have negative functions in malignant transformation. *DGKα* gene expression is positively linked to lung cancer patient survival (Berrar et al., 2005), and reduction of its expression is synergistic with p53 and Ras mutations in colon cancer (McMurray et al., 2008). The identification of FoxO-dependent DGKα regulation further supports a relationship between cell transformation and reduced DGKα expression. A decrease in DGKα levels could lead to a concomitant loss of its DAG-brake functions that, in tumor cells, is linked to the onset of malignant transformation. Lower DGKα levels in transformed cells would switch DGKα requirements to those of PA-mediated regulation of tyrosine kinases and/or atypical PKC.

We found that in breast-derived cells, independent of their untransformed or malignant character, DGK activity and specifically, DGKα levels increased as the cells reached high confluence. This behavior appears to differ from that described by Morita and coworkers (Morita et al., 2009). Using HEK-293 cells, these authors observed that PA levels decrease as a culture becomes more confluent, an effect they attribute to DGKζ. At difference from HEK-293, the cell-cell contacts of breast-derived cell lines can be more robust or differentiated. One type of cell-cell contact is that mediated by cadherins, responsible for adherens junction formation. Cadherins form complexes with the α- and β-catenins, which link cell adhesion molecules to the cytoskeleton. Catenins can also interact with the transcription factor lymphoid enhancer-binding factor 1 (LEF-1) to modulate gene expression (Behrens et al., 1996). Tthe *DGKα* promoter has one LEF-1 site (Martínez-Moreno et al., under revision) and it is likely that, as cells reach confluence, LEF-1 signaling modifies *DGKα* expression.

DGKα might be also needed for the formation and/or maintenance of tight junctions, a specialized type of junction established in endothelia and epithelia. This junction is composed of distinctive integral proteins (claudins and occludins) that bind through cytoplasmic plaque adaptor proteins to the actin cytoskeleton (Gonzalez-

Mariscal et al., 2008). Tight junctions also contribute to the maintenance of the apical and basolateral domains of polarized epithelial cells. Epithelial polarity is controlled by various proteins, among them the PAR complex, which includes Par3, Par6 and aPKCζ. aPKCζ is recruited to subapical Par3-Par6 complexes, and phosphorylates and excludes Par3 from tight junction sites, allowing junction formation and the separation of the apical and subapical domains (Martin-Belmonte and Perez-Moreno, 2012). DGKα-generated PA regulates aPKCζ, to promote cell migration (Chianale et al., 2010). This isoform probably also contributes to other aPKCζ-mediated processes in epithelial cells, such as the establishment of a polarized epithelium.

We also show that DGKα interacts with the ILK. The ILK is a protein that interacts specifically with the β1- and β3-chains of integrins, the major cellular ECM receptors. There is controversy as to whether ILK is a true protein kinase or only a docking pseudokinase protein (Wickstrom et al., 2010). In any case, ILK plays a central role in the transmission of signaling events initiated by cell-matrix interactions (McDonald et al., 2008a). DGKα was recently shown to regulate integrin recycling through PA-dependent RCP regulation (Rainero et al., 2012). These data suggest that DGKα is tightly coupled to the transduction of signals from cell-cell and cell-ECM interactions. We propose a tumor suppressor role for DGKα that helps to preserve the quiescence state and it contributes to the formation and maintenance of cell-cell contacts and epithelial polarization.

This function correlates with the changes in DGKα expression observed in the normal mouse mammary gland. The mammary gland is a complex organ constituted of cells of diverse origins. To prevent contamination by other lineages, especially by lymphoid cells (which express high DGKα levels), we used a flow cytometry cell sorting technique to isolate mammary epithelial cells. In addition, this approach allow us to distinguish between basal and luminal epithelial cells, as well as the different luminal subsets. Age-dependent analysis showed that *DGKα* mRNA levels are higher in luminal than in basal cells; this pattern changed during puberty (10-11 weeks after birth). We also analyzed *DGKα* expression in different luminal compartments, and observed that its expression increased at the onset of puberty. This *DGKα* increase was observed mainly in the double positive (or ER^+) and the alveolar progenitors, whereas levels remained invariable in the non-clonogenic or differentiated luminal population. These data are consistent with those found in array analyses. A GEO database search

(Edgar et al., 2002) (accession GDS2360 and GDS2721) showed that *DGKα* is expressed strongly during puberty and diminishes during pregnancy, lactation and involution (not analyzed here).

We have recently characterized the presence of two independent promoter regions that drive *DGKα* expression (Martínez-Moreno et al., under revision). We found that the most active promoter in mouse mammary epithelial cells is promoter 2. This contrasts with other cell types, such as T cells, in which *DGKα* is driven mainly by promoter 1 (García-Liévana, unpublished observations). *In silico* analysis of *DGKα* promoter 1 showed that it has a STAT-binding site, which is thought to repress the transcription activity of this promoter (Verdeil et al., 2006). The activity of these transcription factors is high in the developing mammary gland (Visvader, 2009). Although further studies are required, these data link *DGKα* expression with the proliferative-differentiation state of the mammary gland.

2. DGKα and DGKζ contribute to the maintenance of lipid metabolism and oncogenic axes in breast cancer

The DGK balance the levels of two lipids that lie at the core of lipid biosynthesis pathways and of the oncogenic axes that help to maintain the malignant phenotype. Further analysis of the DGK contribution to these processes could help to determine whether they function as tumor promoters or suppressors, as well as the mechanisms responsible for this dual behavior.

2.1. DAG consumption helps to maintain the lipogenic metabolism of breast tumors

We first focused on the roles of DGKα and DGKζ in the control of breast cancer lipid metabolism (**Figure 33**). The targeting of tumor metabolism promises new opportunities for cancer treatment. Inhibition of DGK in breast cancer cells caused a chronic increase in DAG levels. As a lipid second messenger, DAG integrates signaling and metabolic functions, which might be a way in which cells join several mechanisms to regulate lipid metabolism. We used a DAG-specific fluorescent probe to show that, in exponentially growing cells, DAG accumulates mainly in the Golgi apparatus. DAG is generated in the Golgi mainly by sphingomyelin synthase action, which transforms ceramide and PtdCho into sphingomyelin and DAG. DGK inhibition causes increased DAG levels in Golgi and in vesicles, and as a result, might alter the morphology of and

distribution of lipids in the Golgi. DAG increase thus promotes PKD activation, which in turn triggers PtdIns-4-phosphate production via PI4K activation (Yeaman et al., 2004). PtdIns-4-phosphate serves as a docking site for several lipid transporters, such as CERT and OSBP (oxysterol-binding protein), responsible respectively for ceramide and oxysterol trafficking from the endoplasmic reticulum to the Golgi. This helps to maintain a continuous lipid flow within the cell compartments. An excessive production of DAG limits lipid transport through PKD hyperactivation, which in turn phosphorylates the CERT and the OSBP PH domains and impairs their binding to membranes (Malhotra and Campelo, 2011). This mechanism by which DAG-dependent regulation of PKD promotes or limits lipid transport from the endoplasmic reticulum to the Golgi helps the cells maintain a correct supply of lipids with important structural functions, such as PtdCho.

An excess of DAG together with lack of PA production helps to explain the profound alteration in Golgi structure that results from DGK inhibition. This phenotype resembles that found after cell treatment with ilimaquinone, a natural metabolite of sponges that vesiculates the Golgi apparatus by overactivating the membrane fission reaction. This reaction implicates DAG accumulation at the neck of the new vesicle. DAG generation triggers PKD localization and dimerization in the *trans*-Golgi network (TGN, (Baron and Malhotra, 2002)). Vesicle fission is terminated by the accumulation of less-stable lipids such as PA. PA is generated in two ways; one involves PKD-mediated activation of the PLD via the Arf family of GTPases, which produces PA from PtdCho hydrolysis. PA can also be produced from DAG by the action of a DGK, a reaction that additionally reduces DAG levels and delocalizes PKD from the TGN (Malhotra and Campelo, 2011). In our experiments using MDA-MB-231 cells, the DAG-dependent functions are likely to be mediated by the PKD2 or PKD3 isoforms, as this cell line lacks PKD1 (Eiseler et al., 2009).

In addition to its function in the control of lipid distribution within the cell, the Golgi is also the site at which several proteins are modified. An example is the transcription factor SREBP-1, whose active form is generated at the Golgi after cleavage by two proteases (Osborne and Espenshade, 2009). We demonstrate the contribution of DGK activity to SREBP-1 activation (**Figure 33**). DGK inhibition can affect SREBP-1 processing through several non-exclusive mechanisms.

a. DGK can affect SREBP-1 processing by controlling DAG signaling effectors (**Figure 33**). We demonstrate that PKC or PKD inhibition leads to an increase in full-

length SREBP-1 levels. Since SREBP-1 controls its own gene expression, it is probable that DAG effectors control its transcriptional activity. SREBP-1 transcription activity depends on its nuclear location, which for most transcription factors is regulated by phosphorylation. This is the case of SREBP-1, whose phosphorylation by AMPK (AMP-activated protein kinase) impairs processing of the full-length form as well as nuclear translocation of the active factor (Li et al., 2011). A similar mechanism might operate when DAG levels increase. Here we show that treatment with DGK inhibitor causes a migration shift of full-length SREBP-1. In addition, cell treatment with inhibitors of PKC or PKD catalytic activity promotes expression of the SREBP-1, thus suggesting that by inhibiting DAG effectors, DGK also promotes SREBP-1 processing and translocation to the nucleus, where it triggers its own transcription.

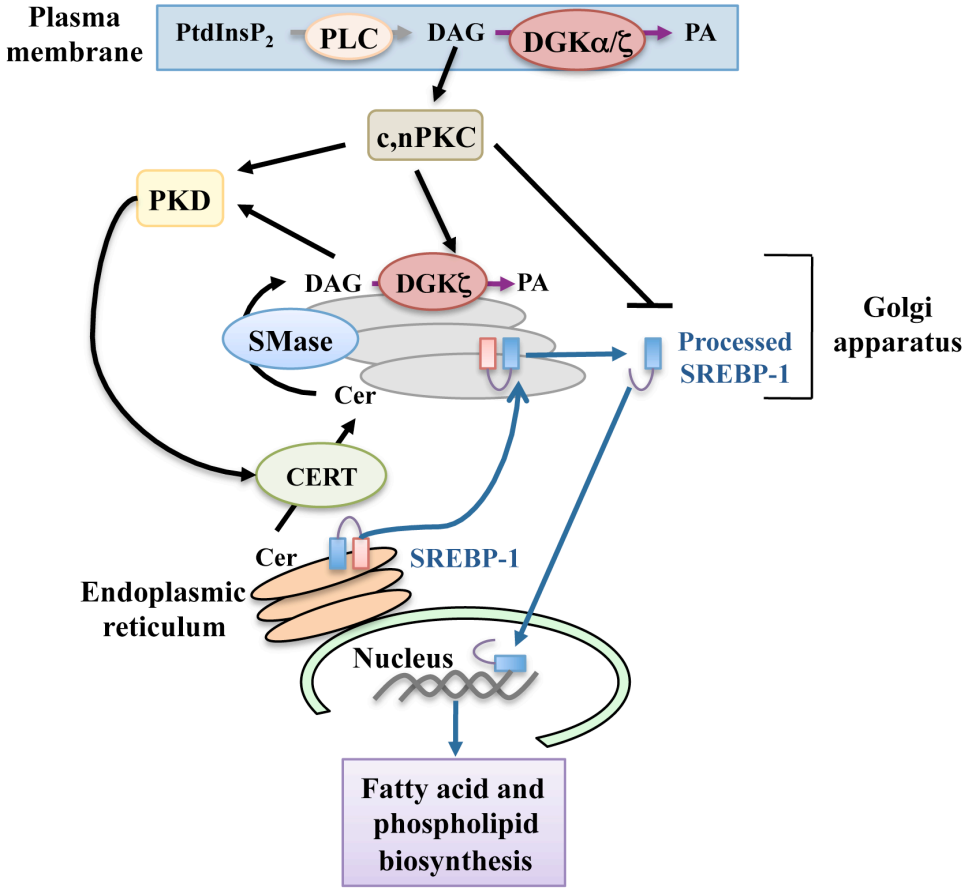


Figure 33. DGK contribution to lipid metabolism in breast cancer cells. DAG can be generated in the plasma membrane after activation of PLC. In the Golgi, DAG is generated mainly from ceramide (Cer) by the action of sphingomyelin synthase (SMase). DAG accumulation directs PKD to the Golgi, where it is further activated by PKC. PKD induces the lipid trafficking from the endoplasmic reticulum to the Golgi apparatus. This promotes the transport of the full-length form of the SREBP-1 transcription factor to the Golgi, where it is processed by proteases. The processed SREBP-1 enters the nucleus, where it activates transcription of its target genes. SREBP-1 phosphorylation, possibly by PKC, prevents this process.

b. By limiting DAG levels, DGK might control the synthesis of several phospholipids, including PtdCho whose cell levels correlate inversely with SREBP-1 processing (Walker et al., 2011). PtdCho can be synthesized directly from DAG; DGK activity therefore limits PtdCho synthesis, promoting SREBP-1 activation.

DGK can also regulate PtdCho indirectly by controlling ceramide transport from the endoplasmic reticulum to the Golgi. Ceramide is transported to the Golgi via the CERT protein, whose PH domain allows it to dock to PtdIns-4-phosphate at the Golgi membrane. Once in the Golgi, ceramide can be transformed into DAG by sphingomyelin synthase, a reaction that consumes PtdCho. DAG accumulation eventually impedes ceramide transport via CERT PKD-dependent phosphorylation, which could lead to PtdCho accumulation.

c. Finally, via regulation of PKD, DGK might control oxysterol transport. PKD-dependent phosphorylation of OSBP inhibits oxysterol transport from the endoplasmic reticulum to the Golgi, leading to oxysterol accumulation at the endoplasmic reticulum. The full-length SREBP-1 protein interacts in the endoplasmic reticulum membrane with SCAP (SREBP cleavage-activating protein). SCAP has a sterol-sensing domain that, when endoplasmic reticulum sterol levels increase, inhibits transport of full-length SREBP to Golgi, impeding its proteolytic processing and activation (Brown et al., 2002). This mechanism operates for both SREBP-1 and -2. Although it is less dependent on DGK activity, SREBP-2 activation is also impaired when DGK is inhibited (not shown).

We found that DGKζ is the principal contributor to this effect. DGKα modulated DAG signaling effectors, but might not regulate DAG metabolism or does so only in very specific contexts. We recently reported that, in the TCR signaling system, DGKζ activity responds to DAG generation (Gharbi et al., 2011). We propose a similar mechanism operating in tumor cells. DGKζ has a MARCKS homology domain that overlaps with a nuclear localization signal. Phosphorylation of this domain by classical PKCα has two consequences, it promotes nuclear export of DGKζ and it triggers DGKζ recruitment to membranes, where the enzyme is active. Several lines of evidence also suggest DGKζ functions in the control of vesicle and protein sorting (Rincon et al., 2011). DGKζ would therefore be more versatile than other DGK for integrating DAG metabolic and signaling functions, which constitute a network, that controls lipid distribution in cells (**Figure 33**).

2.2. PA generation is needed for maintenance of the PI3K-Akt-mTOR axis

DGK not only consume DAG, but also generate PA. Although PA functions are less studied, it is thought that imbalance of PA levels can contribute to progression of diseases such as cancer. Our data indicate that DGK-derived PA contributes to stability and/or activity of both mTOR complexes (**Figure 34**). This concurs with a previous report on the contribution of PA to mTOR activation and complex formation (Toschi et al., 2009). Analysis of various breast cancer cell lines nonetheless showed pronounced differences in the sensitivity of these complexes to DGK and/or rapamycin inhibition. Highly malignant MDA-MB-231 cells maintained significant levels of mTOR/Rictor complex after long-term rapamycin treatment, whereas this association was impaired in the other cell lines. Treatment with R59949 alone had no effect on mTOR complex formation in the MDA-MB-231 line, but disassembled complexes to different extents in the other lines studied. This suggests that the distinct rapamycin sensitivity of these cell lines reflects sensitivity to DGK inhibition, reinforcing the idea that mTOR and DGK operate in the same pathway. The "rapamycin resistance" of MDA-MB-231 cells, evaluated as S6K phosphorylation, is linked to elevated PLD activity (Chen et al., 2003); the lesser sensitivity of these cells to the DGK inhibitor thus suggests that other non-DGK-derived PA pools also contribute to mTOR integrity. In MDA-MB-231 cells, DGK inhibition affects mTOR/Rictor assembly and Akt phosphorylation only in the presence of rapamycin, suggesting an additive effect of the two drugs. DGK-derived PA might also contribute to mTORC2 localization at the membrane. SIN1 (SAPK-interacting protein 1) is an essential component of mTORC2, but not of mTORC1; it helps to maintain the mTOR/Rictor interaction and also interacts with Akt, thus bringing the kinase closer to its substrate (Jacinto et al., 2006). SIN1 has a PH domain that binds several phospholipids, including PA and several PtdIns species (Schroder et al., 2007). DGK-generated PA can thus assist mTORC2 localization at specific cell membranes bearing its substrate, Akt.

Rapamycin-mediated mTORC1 inhibition relieves signals that restrain PI3K activation. Many studies have characterized this negative feedback loop in which mTOR and S6K block further activation of the PI3K-Akt pathway through inhibition of IRS-1 function. Although initially characterized in insulin signaling, this negative feedback is also observed during signaling by other growth factors, including serum (Manning et al., 2005; Parmar et al., 2006). Correct regulation of this loop maintains adequate homeostasis in normal cells, and its triggering contributes to rapamycin

resistance in cancer cells. DGK activity participates in this feedback loop since, as we show, long-term rapamycin treatment leads to DGK activation and concomitant DAG consumption. This observation suggests a role for mTORC1 as a PA sensor, controlling DGK activation to precisely regulate the mTOR-Akt axis. Mitogens such as serum and growth factors promote DGK activation (Avila-Flores et al., 2005; Baldanzi et al., 2004; Schaap et al., 1993) and PA generation (Chen and Fang, 2002; English et al., 1996), which lead to mTORC1 activation mediated by PA binding. In "full PA-binding" conditions, mTORC1 would inhibit DGK activity to restrict mitogenic responses. mTOR and DGK would thus regulate one another, similar to the mTOR interrelation with PI3K (**Figure 34**).

Our results show that, in addition to maintaining adequate mTOR activation, DGK regulates Akt phosphorylation, also through mTOR-independent mechanisms. This is demonstrated in the experiments using rapamycin-treated MCF-7 and MDA-MB-468 cells, in which Akt is phosphorylated in the absence of mTORC2, but is blocked by R59949. This DGK dependence of Akt phosphorylation is enhanced when PI3K activity is upregulated by rapamycin-elicited feedback. DGK inhibition also impaired Akt phosphorylation on Thr308, which is strictly dependent on $PtdInsP_3$ generation, and on PH-based membrane recruitment of Akt and PDK1 (Alessi et al., 1997; McManus et al., 2004).

We suggest that, in transformed cells, PI3K activity depends on DGK metabolic functions. Early studies in fibroblasts showed that Src- or Erbb-dependent transformation led to alteration of a PtdIns kinase activity (later identified as PI3K) accompanied by increased DGK activity (Kato et al., 1987; Sugimoto et al., 1984). This close relationship between the two enzymes might in part be due to the fact that the PI3K substrate $PtdInsP_2$ is generated from DAG through the biochemical pathway referred to as the PtdIns cycle (Jenkins et al., 1994). DGK-catalyzed DAG conversion to PA is the first step in replenishing $PtdInsP_2$, the PLC and PI3K substrate (Bunney and Katan, 2010; Sasaki et al., 2009). Adequate maintenance of this cycle is vital for cell homeostasis, and alterations in PtdIns-modifying enzymes can lead to disease (Wymann and Schneiter, 2008). Our analysis of PtdIns intermediates indicates that blockade of DGK activity drastically reduced PtdIns-4-phosphate and $PtdInsP_2$ concentrations, confirming that DGK activity is needed for maintenance of PtdIns cycle components (**Figure 34**). Low-dose lithium treatment is reported to reduce inositide phosphatase activity and to cause accumulation of PA that is recycled into the PtdIns cycle (Dixon

and Hokin, 1997). Low lithium chloride concentration reversed the effect of the DGK inhibitor, maintaining Akt Ser473 phosphorylation. Our data thus indicate that DGK activity is necessary to maintain fueling of lipid substrates for PI3K.

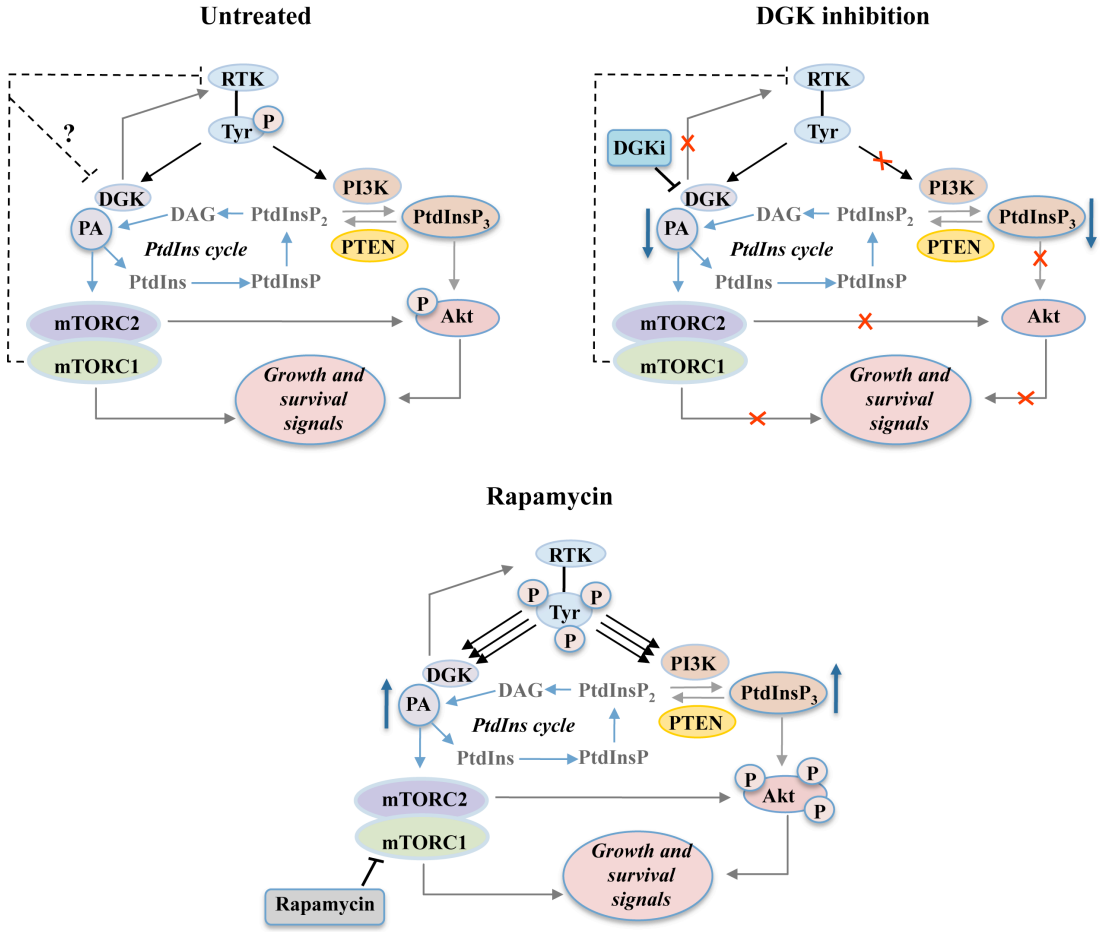


Figure 34. Model of DGK as a key molecule controling breast cancer cell proliferation and survival via the PI3K-Akt-mTOR axis. DGK is activated by mitogens/RTK, probably through phosphotyrosine docking. DGK-derived PA is necessary for correct tyrosine signaling; it activates mTOR and replenishes the PtdIns cycle. PI3K is also activated by phosphotyrosine docking and requires PtdIns recycling to generate $PtdInsP_3$ and activate Akt. In a feedback loop, mTORC1-derived signals restrain phosphotyrosine docking, limiting DGK and PI3K activity (top left). Rapamycin-mediated mTORC1 inhibition relieves this feedback loop, enhancing DGK and PI3K activities (bottom). By acting on these interrelated pathways, DGK controls breast cancer cell proliferation and survival. DGK inhibitor (DGKi) impairs phosphotyrosine signaling as well as PtdIns recycling, blocking PI3K-Akt activation. Tumor lipid metabolism is thus reduced, with a concomitant reduction in growth and survival signals (top right).

In these experiments, we detected no changes in radiolabeled PA. This PA might derive from a non-DGK source, probably PLD, since MDA-MB-231 cells have high PLD activity levels (Chen et al., 2003). PLD-derived PA is reported to accumulate radiolabel in such assays, but at difference from DGK-derived PA, this type of PA does not enter PtdIns resynthesis pathways (Whatmore et al., 1999). DGK-derived PA in

these cells is therefore not essential for mTOR complex formation, as it can be replaced by PLD-derived PA, but it is essential for providing the PI3K substrate.

In addition to the requirement for DGK-derived PA for adequate PtdIns resynthesis and mTOR complex assembly, our data also indicate that DGK activity might control PI3K activation by promoting its binding to RTK (**Figure 34**). Phosphotyrosine-dependent activation of PI3K is needed to relieve the inhibitory function of the regulatory subunit on the catalytic subunit; this is normally abrograted by PKC-mediated Ser/Thr phosphorylation of RTK or one of its scaffold proteins. Although DGK modulate PKC activity, this does not appear to be the mechanism by which DGK inhibition reduces phosphotyrosine-linked PI3K activity in transformed cells.

Using RNAi-mediated knockdown of DGKα and DGKζ, we found isoform-specific functions in the control of the PI3K-Akt-mTOR axis. Both isoforms were able to modulate mTORC1 activity throughout the cell cycle, but only DGKα was implicated in the control of Akt and tyrosine kinase activation. We suggest that DGK-derived PA, as well as that derived from PLD (not shown), would activate mTORC1 directly, as described (Avila-Flores et al., 2005; Chen et al., 2003), reinforcing the concept of the PA-dependence of mTORC1 activity. Inhibition of mTORC1 as a result of DGKζ depletion also helps to explain the DGKζ effect on SREBP-1, since mTORC1 is essential for SREBP-1 activation, and suggests a role for DGKζ in the integrated control of cell growth and metabolism.

A report from our laboratory suggests that class I PI3K products ($PtdInsP_3$ and PtdIns-3,4-P_2) promote DGKα activity (Cipres et al., 2003). In our tumor cell panel, we found the highest DGK activity in those lines with constitutive activation of the PI3K-Akt axis. We also found that DGKα interacted with ILK. As mentioned above, whether ILK has or lacks kinase activity is debated; some studies suggest it is a pseudokinase with scaffold functions (Fukuda et al., 2009), although other reports indicate that it is an atypical Mn^{2+}-dependent kinase that phosphorylates Akt Ser473 in a PI3K-dependent manner (Maydan et al., 2010). ILK interacts with Rictor (McDonald et al., 2008b). Independent of its function, we propose that DGKα is recruited, possibly via ILK, to locations where Akt is expressed, and contributes to the generation of local PA pools that activate the kinase that phosphorylates Akt Ser473 (ILK, Akt, PDK1).

We show that DGKα has a specific function in regulating the non-receptor tyrosine kinase Src; through this mechanism, this isoform might also contribute to the propagation of RTK signaling and Akt activation. Experiments in endothelial cell lines

showed a strong DGKα effect on receptor-mediated tyrosine kinase activity (Baldanzi et al., 2004; Cutrupi et al., 2000). Very little is known about how DGK activity promotes tyrosine phosphorylation; DGK-derived PA might inhibit specific phosphatases (English et al., 1996) or, as in the case of mTOR, stabilize certain interactions. A scaffold role is also possible, as DGKα associates SFK (Baldanzi et al., 2008; Merino et al., 2008). By interacting with SFK, DGKα might relieve the intramolecular inhibitory interactions of Src and activate the protein, as described for other proteins (Playford and Schaller, 2004). DGKα also interacts with the protein tyrosine-phosphatase-interacting protein 51 (PTPI51; (Stenzinger et al., 2009)), a partner of protein tyrosine-phosphatase 1B (PTP1B), which dephosphorylates the SFK C-terminal tyrosine (Tyr 530 in human Src) to promote Src activation. PTP1B is overexpressed in breast tumors with high Src or tyrosine kinase activity (Egan et al., 1999). DGKα-mediated regulation of Src via PTP1B could help to explain the crosstalk between SFK and DGK. Finally, DGK activity might also be critical in the formation of specific membrane compartments, whose distinct lipid composition would allow RTK firing and signal transduction; this hypothesis concurs with data that indicate a role for DGK in the trafficking of clathrin-coated vesicle (Antonescu et al., 2010).

DGKα activity is also regulated by tyrosine phosphorylation. Assay of DGK activity in phosphotyrosine immunoprecipitates shows that it resembles that observed in total cell extracts. Most of our cell lines with an alteration in the PI3K-Akt axis overexpressed RTK or an RTK scaffold. We found that DGK activity (likely to be that of DGKα) is coupled to the HER2 receptor in a $HER2^{+}$ cell line. Increased tyrosine signaling, and possibly PI3K-derived products, might therefore activate DGKα activity.

Current therapies based on the use of rapalogs and PI3K or Akt inhibitors normally relieve feedback loops that trigger RTK and ERK activation (Carracedo et al., 2008; Chandarlapaty et al., 2011; Serra et al., 2011). We show that long-term DGK blockade does not activate ERK, even in the presence of rapamycin. In addition, DGK inhibition or DGKα depletion impairs accumulation of the HIF-1α transcription factor. This protein promotes a transcription program that drives tumor growth in hypoxic conditions, and confers radio- and chemotherapy resistance (Harris, 2002). HIF-1α induction is highly dependent on mTORC1 and mTORC2 activation, reflecting the DGKα contribution to the activity of both complexes.

3. DGK offers new opportunities for breast cancer treatment

Our data show that, by acting on several interrelated pathways, DGK is necessary for breast tumor cell proliferation and survival, and suggest DGK as candidate targets for breast cancer therapy. Inhibition of DGK activity or reduction of DGKζ levels clearly compromised breast cancer cell growth *in vitro*. We found that cell lines with oncogenic mutations in the RTK-PI3K axis (MCF-7 and BT-474) were extremely sensitive to DGK inhibition. In untransformed cells, PI3K activation products are a minimal fraction of PtdIns species, and the $PtdInsP_2$ consumed is rapidly replenished after receptor activation (Hawkins et al., 1992). The oncogenic activation of the RTK-PI3K pathway is quite a different situation, as it requires a continuous supply of PI3K substrates, principally $PtdInsP_2$, whose synthesis is highly PA-dependent. Cancers with PTEN loss of function (e.g., MDA-MB-468) or K-Ras-activating mutations (MDA-MB-231) do not respond well to rapamycin (Weigelt et al., 2011). Our data nonetheless indicate that tumors lacking PTEN are highly sensitive to R59949, and that DGK inhibition prevents oncogenic Akt activation, as well as the Akt activation due to rapamycin-elicited feedback loops in K-Ras mutant tumors. DGK inhibition might thus be an effective chemotherapeutic strategy for the treatment of these especially resistant malignancies.

Inhibition of DGK effectively retarded tumor growth *in vivo*. This effect was more prominent when R59949 was co-administered with rapamycin. We have observed DGK activation following long-term rapamycin treatment, and therefore DGK-derived PA might promote new survival pathways that allow tumor cells to overcome rapamycin effects, including PI3K activation. Together, this renders malignant cells more sensitive to DGK inhibition.

Although its effect on *in vitro* tumor growth was modest, we found that DGKα depletion impaired tumor growth *in vivo*. This might reflect the distinct DGKα requirements for 2D and 3D cell growth. DGKα appears to act locally at sites that normally involve cell-cell and cell-ECM contacts; indeed, *DGKα* mRNA levels increased when cells were grown in 3D matrices *in vitro*. Although tumor cells are assumed to become independent of this signals, ECM trigger proliferation and survival pathways that involve integrin-mediated activation of PI3K and the focal adhesion kinase (FAK)-Src axis (Guo and Giancotti, 2004). Inducible depletion of DGKα in MDA-MB-231 cells abrogated their growth and increased their apoptosis in 3D

matrices, although residual cells were able to grow. This finding reinforces a role for DGKα in the transduction of signals from the extracellular environment. On the other hand, DGKζ-mediated control of the lipogenic metabolism of tumors might be compensated *in vivo* by several factors, for example, DAG hydrolysis by tumor lipases or support of tumor growth by diet fatty acids. This effect was described for monoacylglycerol lipase (MAGL), for which reduced tumor growth was observed only when mice were maintained on a low-fat diet (Nomura et al., 2010).

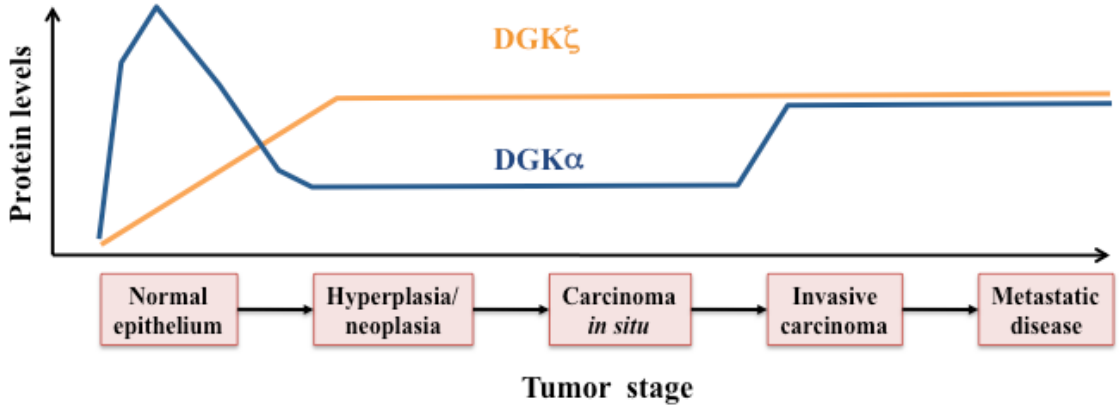


Figure 35. Model for the variation of DGKα and DGKζ levels during transformation of the mammary gland. DGKα levels are high in the normal epithelium and drop during the transformation process, allowing proliferation. DGKα levels must rise to sustain invasion and migration of malignant cells. DGKζ levels increase with transformation and help sustain the lipogenic metabolism of tumors.

Based on our data, we propose a model (**Figure 35**) for the contribution of DGKα and DGKζ to breast cancer progression. In untransformed cells, strong DGKα expression confers a tumor suppressor role on this isoform, probably by maintaining cell-cell contacts and epithelial polarization, and by restricting proliferation. During the transformation process, DGKα levels must drop, although certain levels are maintained and promote tumors by supporting signaling through the RTK-PI3K-Akt axis and contributing to malignant cell migration and invasion (Chianale et al., 2010; Rainero et al., 2012). At this stage, DGKζ would increase or be maintained, to sustain tumor metabolism. This model is reinforced by our preliminary analysis of DGKα and DGKζ expression in human breast tumor-derived samples, in which we found higher DGKα expression, whereas DGKζ levels did not change. Further analysis should nonetheless be done; in addition, higher DGKα levels in breast tumor biopsies might not reflect its expression in the epithelial compartment, as this isoform is highly expressed in cell lineages, such as lymphocytes, that infiltrate the mammary gland during transformation.

Our initial data allow us to propose DGKα and DGKζ as candidates for therapeutic intervention in breast cancer. We anticipate two major advantages of DGK-directed therapy. The first is that long-term DGK inhibition did not activate other oncogenic axes that could lead to resistance to DGK-based therapy. Secondly, we show that both DGK isoforms contribute to tumor progression in breast cancer cells, although their function in the immune system is inhibitory. DGK inhibition might thus retard tumor growth while activating the immune system, an advantage compared to other anti-tumor therapies.

CONCLUSIONS

1. Pharmacological inhibition of DGK blocked tumor growth *in vivo*. The DGK could constitute an efficient drug target for anticancer therapy.

2. Breast cells expressed DGKα and DGKζ, but expression patterns and function differed. DGKα was strongly expressed in non-transformed breast cell lines, whereas DGKζ expression was higher in tumor-derived cell lines. *DGKα* gene expression correlated inversely with FoxO phosphorylation. DGKα levels and activity increased as cells became confluent, suggesting a function in the formation of cell-cell interactions. *DGKα* expression varied during mouse mammary gland development, reaching its highest levels at the onset of puberty.

3. DGK attenuation of DAG metabolism in breast cancer cells was crucial for PKC-PKD activation, maintenance of the Golgi integrity and structure, and correct processing and activation of the SREBP-1 transcription factor. This function was executed mainly by the DGKζ isoform.

4. DGK-generated PA was indispensable for adequate Akt phosphorylation; it controlled the activity and stability of mTOR complexes and supplied the lipid substrates for PI3K activation. DGK activity maintained phosphotyrosine signaling and was coupled to the HER2 receptor. DGKα and DGKζ were upstream regulators of mTORC1, but only DGKα controlled Akt and Src activation.

5. DGK inhibition impaired tumor growth *in vitro* without activating the signaling axes that promote acquisition of resistance. DGK activity was essential for cancer cell survival and was triggered by rapamycin-elicited feedback loops. Cell lines with aberrant activation of the PI3K-Akt-mTOR axis were more sensitive to DGK inhibition.

6. DGKα depletion had a stronger effect on tumor growth *in vivo* than DGKζ depletion. This might be because DGKα was crucial for cell growth in a 3D context, since its expression increased when cells were cultured in 3D matrices and DGKα interacted with ILK, a protein involved in the transduction of signals from the extracellular matrix.

CONCLUSIONES

1. La inhibición farmacológica de las DGK bloquea el crecimiento tumoral *in vivo*. Las DGK podrían considerarse dianas farmacológicas eficientes para la terapia antitumoral.

2. Las células de mama expresan DGKα y DGKζ, pero su patrón de expresión y funcionalidad difieren. La DGKα se expresa altamente en líneas celulares de mama no transformadas, mientras que, la expresión de DGKζ es más altas en líneas tumorales. La expresión del gen de la *DGKα* correlaciona inversamente con la fosforilación de FoxO. Los niveles y actividad de DGKα aumentan conforme las células alcanzan mayor confluencia, sugiriendo una función en la formación de las interacciones célula-célula. La expresión de *DGKα* varía a lo largo del desarrollo de la glándula mamaria murina, alcanzado su máxima expresión en la pubertad.

3. La atenuación del metabolismo del DAG por DGK en células de cáncer de mama es crucial para la activación de PKC-PKD, el mantenimiento de la integridad y estructura del Golgi y, el procesamiento y activación del factor de transcripción SREBP-1. Esta función la ejecuta principalmente la isoforma DGKζ.

4. El PA generado por DGK es clave para la adecuada fosforilación de Akt, controla la actividad y estabilidad de los complejos de mTOR y aporta los sustratos lipídicos para la activación de PI3K. La actividad DGK mantiene la señalización por fosfotirosinas y está acoplada al receptor HER2. Tanto la DGKα como la DGKζ regulan mTORC1, pero sólo la DGKα controla la activación de Akt y Src.

5. La inhibición de DGK impide el crecimiento tumoral *in vitro* sin activar ejes de señalización que proporcionan resistencia. La actividad DGK es esencial para la supervivencia de las células tumorales y se activa por los ejes de retroalimentación desencadenados por la rapamicina. Las líneas celulares con activación aberrante del eje PI3K-Akt-mTOR son más sensibles a la inhibición de DGK.

6. La disminución de DGKα es más potente que la de DGKζ para el crecimiento tumoral *in vivo*. Esto puede deberse a que la DGKα es crucial para el crecimiento celular en un contexto 3D, ya que sus niveles aumentan cuando se cultiva a las células en matrices 3D y la DGKα interacciona con la ILK, una proteína implicada en la transducción de señales de la matriz extracelular.

REFERENCES

Abramovici, H., Mojtabaie, P., Parks, R.J., Zhong, X.P., Koretzky, G.A., Topham, M.K., and Gee, S.H. (2009). Diacylglycerol kinase zeta regulates actin cytoskeleton reorganization through dissociation of Rac1 from RhoGDI. Molecular biology of the cell 20, 2049-2059.

Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R., Reese, C.B., and Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol 7, 261-269.

Antonescu, C.N., Danuser, G., and Schmid, S.L. (2010). Phosphatidic acid plays a regulatory role in clathrin-mediated endocytosis. Molecular biology of the cell 21, 2944-2952.

Assender, J.W., Gee, J.M., Lewis, I., Ellis, I.O., Robertson, J.F., and Nicholson, R.I. (2007). Protein kinase C isoform expression as a predictor of disease outcome on endocrine therapy in breast cancer. Journal of clinical pathology 60, 1216-1221.

Avila-Flores, A., Santos, T., Rincon, E., and Merida, I. (2005). Modulation of the mammalian target of rapamycin pathway by diacylglycerol kinase-produced phosphatidic acid. The Journal of biological chemistry 280, 10091-10099.

Bacchiocchi, R., Baldanzi, G., Carbonari, D., Capomagi, C., Colombo, E., van Blitterswijk, W.J., Graziani, A., and Fazioli, F. (2005). Activation of alpha-diacylglycerol kinase is critical for the mitogenic properties of anaplastic lymphoma kinase. Blood 106, 2175-2182.

Baldanzi, G., Cutrupi, S., Chianale, F., Gnocchi, V., Rainero, E., Porporato, P., Filigheddu, N., van Blitterswijk, W.J., Parolini, O., Bussolino, F., Sinigaglia, F., and Graziani, A. (2008). Diacylglycerol kinase-alpha phosphorylation by Src on Y335 is required for activation, membrane recruitment and Hgf-induced cell motility. Oncogene 27, 942-956.

Baldanzi, G., Mitola, S., Cutrupi, S., Filigheddu, N., van Blitterswijk, W.J., Sinigaglia, F., Bussolino, F., and Graziani, A. (2004). Activation of diacylglycerol kinase alpha is required for VEGF-induced angiogenic signaling in vitro. Oncogene 23, 4828-4838.

Baron, C.L., and Malhotra, V. (2002). Role of diacylglycerol in PKD recruitment to the TGN and protein transport to the plasma membrane. Science 295, 325-328.

Behrens, J., von Kries, J.P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. Nature 382, 638-642.

Benjamin, D., Colombi, M., Moroni, C., and Hall, M.N. (2011). Rapamycin passes the torch: a new generation of mTOR inhibitors. Nature reviews. Drug discovery 10, 868-880.

Berrar, D., Sturgeon, B., Bradbury, I., Downes, C.S., and Dubitzky, W. (2005). Survival trees for analyzing clinical outcome in lung adenocarcinomas based on gene expression profiles: identification of neogenin and diacylglycerol kinase alpha expression as critical factors. J Comput Biol 12, 534-544.

Bligh, E.G., and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37, 911-917.

Breuleux, M., Klopfenstein, M., Stephan, C., Doughty, C.A., Barys, L., Maira, S.M., Kwiatkowski, D., and Lane, H.A. (2009). Increased AKT S473 phosphorylation after mTORC1 inhibition is rictor dependent and does not predict tumor cell response to PI3K/mTOR inhibition. Mol Cancer Ther 8, 742-753.

Brisken, C., and O'Malley, B. (2010). Hormone action in the mammary gland. Cold Spring Harbor perspectives in biology 2, a003178.

Brown, A.J., Sun, L., Feramisco, J.D., Brown, M.S., and Goldstein, J.L. (2002). Cholesterol addition to ER membranes alters conformation of SCAP, the SREBP escort protein that regulates cholesterol metabolism. Mol Cell 10, 237-245.

Bunney, T.D., and Katan, M. (2010). Phosphoinositide signalling in cancer: beyond PI3K and PTEN. Nat Rev Cancer 10, 342-352.

Cai, J., Abramovici, H., Gee, S.H., and Topham, M.K. (2009). Diacylglycerol kinases as sources of phosphatidic acid. Biochimica et biophysica acta 1791, 942-948.

Carracedo, A., Ma, L., Teruya-Feldstein, J., Rojo, F., Salmena, L., Alimonti, A., Egia, A., Sasaki, A.T., Thomas, G., Kozma, S.C., Papa, A., Nardella, C., Cantley, L.C., Baselga, J., and Pandolfi, P.P. (2008). Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. The Journal of clinical investigation 118, 3065-3074.

Carrasco, S., and Merida, I. (2004). Diacylglycerol-dependent binding recruits PKCtheta and RasGRP1 C1 domains to specific subcellular localizations in living T lymphocytes. Molecular biology of the cell 15, 2932-2942.

Carrasco, S., and Merida, I. (2007). Diacylglycerol, when simplicity becomes complex. Trends in biochemical sciences 32, 27-36.

Carter, H., Samayoa, J., Hruban, R.H., and Karchin, R. (2010). Prioritization of driver mutations in pancreatic cancer using cancer-specific high-throughput annotation of somatic mutations (CHASM). Cancer biology & therapy 10, 582-587.

Cenni, V., Doppler, H., Sonnenburg, E.D., Maraldi, N., Newton, A.C., and Toker, A. (2002). Regulation of novel protein kinase C epsilon by phosphorylation. The Biochemical journal 363, 537-545.

Chalhoub, N., Zhu, G., Zhu, X., and Baker, S.J. (2009). Cell type specificity of PI3K signaling in Pdk1- and Pten-deficient brains. Genes Dev 23, 1619-1624.

Chandarlapaty, S., Sawai, A., Scaltriti, M., Rodrik-Outmezguine, V., Grbovic-Huezo, O., Serra, V., Majumder, P.K., Baselga, J., and Rosen, N. (2011). AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity. Cancer Cell 19, 58-71.

Chen, J., and Fang, Y. (2002). A novel pathway regulating the mammalian target of rapamycin (mTOR) signaling. Biochemical pharmacology 64, 1071-1077.

Chen, Y., Rodrik, V., and Foster, D.A. (2005). Alternative phospholipase D/mTOR survival signal in human breast cancer cells. Oncogene 24, 672-679.

Chen, Y., Zheng, Y., and Foster, D.A. (2003). Phospholipase D confers rapamycin resistance in human breast cancer cells. Oncogene 22, 3937-3942.

Chianale, F., Rainero, E., Cianflone, C., Bettio, V., Pighini, A., Porporato, P.E., Filigheddu, N., Serini, G., Sinigaglia, F., Baldanzi, G., and Graziani, A. (2010). Diacylglycerol kinase alpha mediates HGF-induced Rac activation and membrane ruffling by regulating atypical PKC and RhoGDI. Proceedings of the National Academy of Sciences of the United States of America 107, 4182-4187.

Chibalin, A.V., Leng, Y., Vieira, E., Krook, A., Bjornholm, M., Long, Y.C., Kotova, O., Zhong, Z., Sakane, F., Steiler, T., Nylen, C., Wang, J., Laakso, M., Topham, M.K., Gilbert, M., Wallberg-Henriksson, H., and Zierath, J.R. (2008). Downregulation of diacylglycerol kinase delta contributes to hyperglycemia-induced insulin resistance. Cell 132, 375-386.

Cipres, A., Carrasco, S., Merino, E., Diaz, E., Krishna, U.M., Falck, J.R., Martinez, A.C., and Merida, I. (2003). Regulation of diacylglycerol kinase alpha by phosphoinositide 3-kinase lipid products. The Journal of biological chemistry 278, 35629-35635.

Corbalan-Garcia, S., Sanchez-Carrillo, S., Garcia-Garcia, J., and Gomez-Fernandez, J.C. (2003). Characterization of the membrane binding mode of the C2 domain of PKC epsilon. Biochemistry 42, 11661-11668.

Cutrupi, S., Baldanzi, G., Gramaglia, D., Maffe, A., Schaap, D., Giraudo, E., van Blitterswijk, W., Bussolino, F., Comoglio, P.M., and Graziani, A. (2000). Src-mediated activation of alpha-diacylglycerol kinase is required for hepatocyte growth factor-induced cell motility. The EMBO journal 19, 4614-4622.

DeBerardinis, R.J., Lum, J.J., Hatzivassiliou, G., and Thompson, C.B. (2008). The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. Cell metabolism 7, 11-20.

Debnath, J., Muthuswamy, S.K., and Brugge, J.S. (2003). Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods 30, 256-268.

Dixon, J.F., and Hokin, L.E. (1997). The antibipolar drug valproate mimics lithium in stimulating glutamate release and inositol 1,4,5-trisphosphate accumulation in brain cortex slices but not accumulation of inositol monophosphates and bisphosphates. Proceedings of the National Academy of Sciences of the United States of America 94, 4757-4760.

Dobrosotskaya, I.Y., Seegmiller, A.C., Brown, M.S., Goldstein, J.L., and Rawson, R.B. (2002). Regulation of SREBP processing and membrane lipid production by phospholipids in Drosophila. Science 296, 879-883.

Edgar, R., Domrachev, M., and Lash, A.E. (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 30, 207-210.

Egan, C., Pang, A., Durda, D., Cheng, H.C., Wang, J.H., and Fujita, D.J. (1999). Activation of Src in human breast tumor cell lines: elevated levels of phosphotyrosine phosphatase activity that preferentially recognizes the Src carboxy terminal negative regulatory tyrosine 530. Oncogene 18, 1227-1237.

Eiseler, T., Doppler, H., Yan, I.K., Goodison, S., and Storz, P. (2009). Protein kinase D1 regulates matrix metalloproteinase expression and inhibits breast cancer cell invasion. Breast Cancer Res 11, R13.

English, D., Cui, Y., and Siddiqui, R.A. (1996). Messenger functions of phosphatidic acid. Chem Phys Lipids 80, 117-132.

Eroles, P., Bosch, A., Alejandro Perez-Fidalgo, J., and Lluch, A. (2011). Molecular biology in breast cancer: Intrinsic subtypes and signaling pathways. Cancer treatment reviews.

Evangelisti, C., Astolfi, A., Gaboardi, G.C., Tazzari, P., Pession, A., Goto, K., and Martelli, A.M. (2009). TIS21/BTG2/PC3 and cyclin D1 are key determinants of nuclear diacylglycerol kinase-zeta-dependent cell cycle arrest. Cellular signalling 21, 801-809.

Fang, Y., Vilella-Bach, M., Bachmann, R., Flanigan, A., and Chen, J. (2001). Phosphatidic acid-mediated mitogenic activation of mTOR signaling. Science 294, 1942-1945.

Filigheddu, N., Sampietro, S., Chianale, F., Porporato, P.E., Gaggianesi, M., Gregnanin, I., Rainero, E., Ferrara, M., Perego, B., Riboni, F., Baldanzi, G., Graziani, A., and Surico, N. (2011). Diacylglycerol kinase alpha mediates 17-beta-estradiol-induced proliferation, motility, and anchorage-independent growth of Hec-1A endometrial cancer cell line through the G protein-coupled estrogen receptor GPR30. Cellular signalling 23, 1988-1996.

Flores, I., Casaseca, T., Martinez, A.C., Kanoh, H., and Merida, I. (1996). Phosphatidic acid generation through interleukin 2 (IL-2)-induced alpha-diacylglycerol kinase activation is an essential step in IL-2-mediated lymphocyte proliferation. The Journal of biological chemistry 271, 10334-10340.

Fu, Y., and Rubin, C.S. (2011). Protein kinase D: coupling extracellular stimuli to the regulation of cell physiology. EMBO Rep 12, 785-796.

Fujikawa, K., Imai, S., Sakane, F., and Kanoh, H. (1993). Isolation and characterization of the human diacylglycerol kinase gene. The Biochemical journal 294 (Pt 2), 443-449.

Fukuda, K., Gupta, S., Chen, K., Wu, C., and Qin, J. (2009). The pseudoactive site of ILK is essential for its binding to alpha-Parvin and localization to focal adhesions. Mol Cell 36, 819-830.

Gharbi, S.I., Rincon, E., Avila-Flores, A., Torres-Ayuso, P., Almena, M., Cobos, M.A., Albar, J.P., and Merida, I. (2011). Diacylglycerol kinase zeta controls diacylglycerol metabolism at the immunological synapse. Molecular biology of the cell 22, 4406-4414.

Ghosh, S., Strum, J.C., Sciorra, V.A., Daniel, L., and Bell, R.M. (1996). Raf-1 kinase possesses distinct binding domains for phosphatidylserine and phosphatidic acid. Phosphatidic acid regulates the translocation of Raf-1 in 12-O-tetradecanoylphorbol-13-acetate-stimulated Madin-Darby canine kidney cells. The Journal of biological chemistry 271, 8472-8480.

Gonzalez-Mariscal, L., Tapia, R., and Chamorro, D. (2008). Crosstalk of tight junction components with signaling pathways. Biochimica et biophysica acta 1778, 729-756.

Griner, E.M., and Kazanietz, M.G. (2007). Protein kinase C and other diacylglycerol effectors in cancer. Nat Rev Cancer 7, 281-294.

Gu, J., and Iyer, V.R. (2006). PI3K signaling and miRNA expression during the response of quiescent human fibroblasts to distinct proliferative stimuli. Genome Biol 7, R42.

Guertin, D.A., and Sabatini, D.M. (2007). Defining the role of mTOR in cancer. Cancer Cell 12, 9-22.

Guo, W., and Giancotti, F.G. (2004). Integrin signalling during tumour progression. Nat Rev Mol Cell Biol 5, 816-826.

Hao, X., Sun, B., Hu, L., Lahdesmaki, H., Dunmire, V., Feng, Y., Zhang, S.W., Wang, H., Wu, C., Wang, H., Fuller, G.N., Symmans, W.F., Shmulevich, I., and Zhang, W. (2004). Differential gene and protein expression in primary breast malignancies and their lymph node metastases as revealed by combined cDNA microarray and tissue microarray analysis. Cancer 100, 1110-1122.

Harrington, L.S., Findlay, G.M., and Lamb, R.F. (2005). Restraining PI3K: mTOR signalling goes back to the membrane. Trends in biochemical sciences 30, 35-42.

Harris, A.L. (2002). Hypoxia--a key regulatory factor in tumour growth. Nat Rev Cancer 2, 38-47.

Hawkins, P.T., Jackson, T.R., and Stephens, L.R. (1992). Platelet-derived growth factor stimulates synthesis of PtdIns(3,4,5)P3 by activating a PtdIns(4,5)P2 3-OH kinase. Nature 358, 157-159.

Hietakangas, V., and Cohen, S.M. (2007). Re-evaluating AKT regulation: role of TOR complex 2 in tissue growth. Genes Dev 21, 632-637.

Hokin, L.E., and Hokin, M.R. (1959). Diglyceride phosphokinase: an enzyme which catalyzes the synthesis of phosphatidic acid. Biochimica et biophysica acta 31, 285-287.

Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S.Y., Huang, Q., Qin, J., and Su, B. (2006). SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. Cell 127, 125-137.

Jenkins, G.H., Fisette, P.L., and Anderson, R.A. (1994). Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid. The Journal of biological chemistry 269, 11547-11554.

Jiang, Y., Sakane, F., Kanoh, H., and Walsh, J.P. (2000). Selectivity of the diacylglycerol kinase inhibitor 3-[2-(4-[bis-(4-fluorophenyl)methylene]-1-piperidinyl)ethyl]-2, 3-dihydro-2-thioxo-4(1H)quinazolinone (R59949) among diacylglycerol kinase subtypes. Biochemical pharmacology 59, 763-772.

Jones, D.R., Gonzalez-Garcia, A., Diez, E., Martinez, A.C., Carrera, A.C., and Merida, I. (1999). The identification of phosphatidylinositol 3,5-bisphosphate in T-lymphocytes and its regulation by interleukin-2. The Journal of biological chemistry 274, 18407-18413.

Kai, M., Yasuda, S., Imai, S., Toyota, M., Kanoh, H., and Sakane, F. (2009). Diacylglycerol kinase alpha enhances protein kinase Czeta-dependent phosphorylation at Ser311 of p65/RelA subunit of nuclear factor-kappaB. FEBS letters 583, 3265-3268.

Kannan, K., Amariglio, N., Rechavi, G., Jakob-Hirsch, J., Kela, I., Kaminski, N., Getz, G., Domany, E., and Givol, D. (2001). DNA microarrays identification of primary and secondary target genes regulated by p53. Oncogene 20, 2225-2234.

Kanoh, H., Yamada, K., and Sakane, F. (1990). Diacylglycerol kinase: a key modulator of signal transduction? Trends in biochemical sciences 15, 47-50.

Kato, M., Kawai, S., and Takenawa, T. (1987). Altered signal transduction in erbB-transformed cells. Implication of enhanced inositol phospholipid metabolism in erbB-induced transformation. The Journal of biological chemistry 262, 5696-5704.

Kenny, P.A., Lee, G.Y., Myers, C.A., Neve, R.M., Semeiks, J.R., Spellman, P.T., Lorenz, K., Lee, E.H., Barcellos-Hoff, M.H., Petersen, O.W., Gray, J.W., and Bissell, M.J. (2007). The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. Molecular oncology 1, 84-96.

Kim, D.H., Sarbassov, D.D., Ali, S.M., King, J.E., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell 110, 163-175.

Krycer, J.R., Sharpe, L.J., Luu, W., and Brown, A.J. (2010). The Akt-SREBP nexus: cell signaling meets lipid metabolism. Trends in endocrinology and metabolism: TEM 21, 268-276.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Laplante, M., and Sabatini, D.M. (2009). mTOR signaling at a glance. Journal of cell science 122, 3589-3594.

Li, Y., Xu, S., Mihaylova, M.M., Zheng, B., Hou, X., Jiang, B., Park, O., Luo, Z., Lefai, E., Shyy, J.Y., Gao, B., Wierzbicki, M., Verbeuren, T.J., Shaw, R.J., Cohen, R.A., and Zang, M. (2011). AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. Cell metabolism 13, 376-388.

Loewen, C.J. (2012). Lipids as conductors in the orchestra of life. F1000 biology reports 4, 4.

Lonne, G.K., Cornmark, L., Zahirovic, I.O., Landberg, G., Jirstrom, K., and Larsson, C. (2010). PKCalpha expression is a marker for breast cancer aggressiveness. Molecular cancer 9, 76.

Los, A.P., de Widt, J., Topham, M.K., van Blitterswijk, W.J., and Divecha, N. (2007). Protein kinase C inhibits binding of diacylglycerol kinase-zeta to the retinoblastoma protein. Biochimica et biophysica acta 1773, 352-357.

Luo, B., Prescott, S.M., and Topham, M.K. (2003a). Association of diacylglycerol kinase zeta with protein kinase C alpha: spatial regulation of diacylglycerol signaling. The Journal of cell biology 160, 929-937.

Luo, B., Prescott, S.M., and Topham, M.K. (2003b). Protein kinase C alpha phosphorylates and negatively regulates diacylglycerol kinase zeta. The Journal of biological chemistry 278, 39542-39547.

Luo, B., Prescott, S.M., and Topham, M.K. (2004). Diacylglycerol kinase zeta regulates phosphatidylinositol 4-phosphate 5-kinase Ialpha by a novel mechanism. Cellular signalling 16, 891-897.

Malhotra, V., and Campelo, F. (2011). PKD regulates membrane fission to generate TGN to cell surface transport carriers. Cold Spring Harbor perspectives in biology 3.

Manning, B.D., and Cantley, L.C. (2007). AKT/PKB signaling: navigating downstream. Cell 129, 1261-1274.

Manning, B.D., Logsdon, M.N., Lipovsky, A.I., Abbott, D., Kwiatkowski, D.J., and Cantley, L.C. (2005). Feedback inhibition of Akt signaling limits the growth of tumors lacking Tsc2. Genes Dev 19, 1773-1778.

Marchet, A., Mocellin, S., Belluco, C., Ambrosi, A., DeMarchi, F., Mammano, E., Digito, M., Leon, A., D'Arrigo, A., Lise, M., and Nitti, D. (2007). Gene expression profile of primary gastric cancer: towards the prediction of lymph node status. Annals of surgical oncology 14, 1058-1064.

Martin-Belmonte, F., and Perez-Moreno, M. (2012). Epithelial cell polarity, stem cells and cancer. Nat Rev Cancer 12, 23-38.

Maydan, M., McDonald, P.C., Sanghera, J., Yan, J., Rallis, C., Pinchin, S., Hannigan, G.E., Foster, L.J., Ish-Horowicz, D., Walsh, M.P., and Dedhar, S. (2010). Integrin-linked kinase is a functional Mn2+-dependent protein kinase that regulates glycogen synthase kinase-3beta (GSK-3beta) phosphorylation. PLoS One 5, e12356.

McDonald, P.C., Fielding, A.B., and Dedhar, S. (2008a). Integrin-linked kinase--essential roles in physiology and cancer biology. Journal of cell science 121, 3121-3132.

McDonald, P.C., Oloumi, A., Mills, J., Dobreva, I., Maidan, M., Gray, V., Wederell, E.D., Bally, M.B., Foster, L.J., and Dedhar, S. (2008b). Rictor and integrin-linked kinase interact and regulate Akt phosphorylation and cancer cell survival. Cancer Res 68, 1618-1624.

McManus, E.J., Collins, B.J., Ashby, P.R., Prescott, A.R., Murray-Tait, V., Armit, L.J., Arthur, J.S., and Alessi, D.R. (2004). The in vivo role of PtdIns(3,4,5)P3 binding to PDK1 PH domain defined by knockin mutation. The EMBO journal 23, 2071-2082.

McMurray, H.R., Sampson, E.R., Compitello, G., Kinsey, C., Newman, L., Smith, B., Chen, S.R., Klebanov, L., Salzman, P., Yakovlev, A., and Land, H. (2008). Synergistic response to oncogenic mutations defines gene class critical to cancer phenotype. Nature 453, 1112-1116.

Menendez, J.A., and Lupu, R. (2007). Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. Nat Rev Cancer 7, 763-777.

Merida, I., Avila-Flores, A., Garcia, J., Merino, E., Almena, M., and Torres-Ayuso, P. (2009). Diacylglycerol kinase alpha, from negative modulation of T cell activation to control of cancer progression. Advances in enzyme regulation 49, 174-188.

Merida, I., Avila-Flores, A., and Merino, E. (2008). Diacylglycerol kinases: at the hub of cell signalling. The Biochemical journal 409, 1-18.

Merino, E., Avila-Flores, A., Shirai, Y., Moraga, I., Saito, N., and Merida, I. (2008). Lck-dependent tyrosine phosphorylation of diacylglycerol kinase alpha regulates its membrane association in T cells. J Immunol 180, 5805-5815.

Meyer, G., Gicklhorn, D., Strive, T., Radsak, K., and Eickmann, M. (2002). A three-residue signal confers localization of a reporter protein in the inner nuclear membrane. Biochemical and biophysical research communications 291, 966-971.

Mills, G.B., Jurisica, I., Yarden, Y., and Norman, J.C. (2009). Genomic amplicons target vesicle recycling in breast cancer. The Journal of clinical investigation 119, 2123-2127.

Milne, S.B., Ivanova, P.T., Armstrong, M.D., Myers, D.S., Lubarda, J., Shulga, Y.V., Topham, M.K., Brown, H.A., and Epand, R.M. (2008). Dramatic differences in the roles in lipid metabolism of two isoforms of diacylglycerol kinase. Biochemistry 47, 9372-9379.

Morita, S.Y., Ueda, K., and Kitagawa, S. (2009). Enzymatic measurement of phosphatidic acid in cultured cells. Journal of lipid research 50, 1945-1952.

Nelson, C.D., Perry, S.J., Regier, D.S., Prescott, S.M., Topham, M.K., and Lefkowitz, R.J. (2007). Targeting of diacylglycerol degradation to M1 muscarinic receptors by beta-arrestins. Science 315, 663-666.

Nelson, C.M., and Bissell, M.J. (2006). Of extracellular matrix, scaffolds, and signaling: tissue architecture regulates development, homeostasis, and cancer. Annual review of cell and developmental biology 22, 287-309.

Neve, R.M., Chin, K., Fridlyand, J., Yeh, J., Baehner, F.L., Fevr, T., Clark, L., Bayani, N., Coppe, J.P., Tong, F., Speed, T., Spellman, P.T., DeVries, S., Lapuk, A., Wang, N.J., Kuo, W.L., Stilwell, J.L., Pinkel, D., Albertson, D.G., Waldman, F.M., McCormick, F., Dickson, R.B., Johnson, M.D., Lippman, M., Ethier, S., Gazdar, A., and Gray, J.W. (2006). A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 10, 515-527.

Nomura, D.K., Long, J.Z., Niessen, S., Hoover, H.S., Ng, S.W., and Cravatt, B.F. (2010). Monoacylglycerol lipase regulates a fatty acid network that promotes cancer pathogenesis. Cell 140, 49-61.

Olenchock, B.A., Guo, R., Carpenter, J.H., Jordan, M., Topham, M.K., Koretzky, G.A., and Zhong, X.P. (2006). Disruption of diacylglycerol metabolism impairs the induction of T cell anergy. Nature immunology 7, 1174-1181.

Osborne, T.F., and Espenshade, P.J. (2009). Evolutionary conservation and adaptation in the mechanism that regulates SREBP action: what a long, strange tRIP it's been. Genes Dev 23, 2578-2591.

Pan, Q., Bao, L.W., Kleer, C.G., Sabel, M.S., Griffith, K.A., Teknos, T.N., and Merajver, S.D. (2005). Protein kinase C epsilon is a predictive biomarker of aggressive breast cancer and a validated target for RNA interference anticancer therapy. Cancer Res 65, 8366-8371.

Parmar, K.M., Larman, H.B., Dai, G., Zhang, Y., Wang, E.T., Moorthy, S.N., Kratz, J.R., Lin, Z., Jain, M.K., Gimbrone, M.A., Jr., and Garcia-Cardena, G. (2006).

Integration of flow-dependent endothelial phenotypes by Kruppel-like factor 2. The Journal of clinical investigation 116, 49-58.

Peretti, D., Dahan, N., Shimoni, E., Hirschberg, K., and Lev, S. (2008). Coordinated lipid transfer between the endoplasmic reticulum and the Golgi complex requires the VAP proteins and is essential for Golgi-mediated transport. Molecular biology of the cell 19, 3871-3884.

Playford, M.P., and Schaller, M.D. (2004). The interplay between Src and integrins in normal and tumor biology. Oncogene 23, 7928-7946.

Preiss, J., Loomis, C.R., Bishop, W.R., Stein, R., Niedel, J.E., and Bell, R.M. (1986). Quantitative measurement of sn-1,2-diacylglycerols present in platelets, hepatocytes, and ras- and sis-transformed normal rat kidney cells. The Journal of biological chemistry 261, 8597-8600.

Rainero, E., Caswell, P.T., Muller, P.A., Grindlay, J., McCaffrey, M.W., Zhang, Q., Wakelam, M.J., Vousden, K.H., Graziani, A., and Norman, J.C. (2012). Diacylglycerol kinase alpha controls RCP-dependent integrin trafficking to promote invasive migration. The Journal of cell biology 196, 277-295.

Regier, D.S., Higbee, J., Lund, K.M., Sakane, F., Prescott, S.M., and Topham, M.K. (2005). Diacylglycerol kinase iota regulates Ras guanyl-releasing protein 3 and inhibits Rap1 signaling. Proceedings of the National Academy of Sciences of the United States of America 102, 7595-7600.

Rincon, E., Gharbi, S.I., Santos-Mendoza, T., and Merida, I. (2012). Diacylglycerol kinase zeta: at the crossroads of lipid signaling and protein complex organization. Progress in lipid research 51, 1-10.

Rincon, E., Saez de Guinoa, J., Gharbi, S.I., Sorzano, C.O., Carrasco, Y.R., and Merida, I. (2011). Translocation dynamics of sorting nexin 27 in activated T cells. Journal of cell science 124, 776-788.

Rincon, E., Santos, T., Avila-Flores, A., Albar, J.P., Lalioti, V., Lei, C., Hong, W., and Merida, I. (2007). Proteomics identification of sorting nexin 27 as a diacylglycerol kinase zeta-associated protein: new diacylglycerol kinase roles in endocytic recycling. Molecular & cellular proteomics : MCP 6, 1073-1087.

Rizzo, M.A., Shome, K., Vasudevan, C., Stolz, D.B., Sung, T.C., Frohman, M.A., Watkins, S.C., and Romero, G. (1999). Phospholipase D and its product, phosphatidic acid, mediate agonist-dependent raf-1 translocation to the plasma membrane and the activation of the mitogen-activated protein kinase pathway. The Journal of biological chemistry 274, 1131-1139.

Rizzo, M.A., Shome, K., Watkins, S.C., and Romero, G. (2000). The recruitment of Raf-1 to membranes is mediated by direct interaction with phosphatidic acid and is independent of association with Ras. The Journal of biological chemistry 275, 23911-23918.

Rosse, C., Linch, M., Kermorgant, S., Cameron, A.J., Boeckeler, K., and Parker, P.J. (2010). PKC and the control of localized signal dynamics. Nat Rev Mol Cell Biol 11, 103-112.

Saal, L.H., Holm, K., Maurer, M., Memeo, L., Su, T., Wang, X., Yu, J.S., Malmstrom, P.O., Mansukhani, M., Enoksson, J., Hibshoosh, H., Borg, A., and Parsons, R. (2005). PIK3CA mutations correlate with hormone receptors, node metastasis, and ERBB2, and are mutually exclusive with PTEN loss in human breast carcinoma. Cancer Res 65, 2554-2559.

Sanjuan, M.A., Jones, D.R., Izquierdo, M., and Merida, I. (2001). Role of diacylglycerol kinase alpha in the attenuation of receptor signaling. The Journal of cell biology 153, 207-220.

Sanjuan, M.A., Pradet-Balade, B., Jones, D.R., Martinez, A.C., Stone, J.C., Garcia-Sanz, J.A., and Merida, I. (2003). T cell activation in vivo targets diacylglycerol kinase alpha to the membrane: a novel mechanism for Ras attenuation. J Immunol 170, 2877-2883.

Santos, T., Carrasco, S., Jones, D.R., Merida, I., and Eguinoa, A. (2002). Dynamics of diacylglycerol kinase zeta translocation in living T-cells. Study of the structural domain requirements for translocation and activity. The Journal of biological chemistry 277, 30300-30309.

Sarbassov, D.D., Ali, S.M., Sengupta, S., Sheen, J.H., Hsu, P.P., Bagley, A.F., Markhard, A.L., and Sabatini, D.M. (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol Cell 22, 159-168.

Sasaki, T., Takasuga, S., Sasaki, J., Kofuji, S., Eguchi, S., Yamazaki, M., and Suzuki, A. (2009). Mammalian phosphoinositide kinases and phosphatases. Progress in lipid research 48, 307-343.

Schaap, D., van der Wal, J., van Blitterswijk, W.J., van der Bend, R.L., and Ploegh, H.L. (1993). Diacylglycerol kinase is phosphorylated in vivo upon stimulation of the epidermal growth factor receptor and serine/threonine kinases, including protein kinase C-epsilon. The Biochemical journal 289 (Pt 3), 875-881.

Schroder, W.A., Buck, M., Cloonan, N., Hancock, J.F., Suhrbier, A., Sculley, T., and Bushell, G. (2007). Human Sin1 contains Ras-binding and pleckstrin homology domains and suppresses Ras signalling. Cellular signalling 19, 1279-1289.

Serra, V., Scaltriti, M., Prudkin, L., Eichhorn, P.J., Ibrahim, Y.H., Chandarlapaty, S., Markman, B., Rodriguez, O., Guzman, M., Rodriguez, S., Gili, M., Russillo, M., Parra, J.L., Singh, S., Arribas, J., Rosen, N., and Baselga, J. (2011). PI3K inhibition results in enhanced HER signaling and acquired ERK dependency in HER2-overexpressing breast cancer. Oncogene 30, 2547-2557.

Shin, J.J., and Loewen, C.J. (2011). Putting the pH into phosphatidic acid signaling. BMC Biol 9, 85.

Stace, C.L., and Ktistakis, N.T. (2006). Phosphatidic acid- and phosphatidylserine-binding proteins. Biochimica et biophysica acta 1761, 913-926.

Stemke-Hale, K., Gonzalez-Angulo, A.M., Lluch, A., Neve, R.M., Kuo, W.L., Davies, M., Carey, M., Hu, Z., Guan, Y., Sahin, A., Symmans, W.F., Pusztai, L., Nolden, L.K., Horlings, H., Berns, K., Hung, M.C., van de Vijver, M.J., Valero, V., Gray, J.W., Bernards, R., Mills, G.B., and Hennessy, B.T. (2008). An integrative genomic and

proteomic analysis of PIK3CA, PTEN, and AKT mutations in breast cancer. Cancer Res 68, 6084-6091.

Stenzinger, A., Schreiner, D., Koch, P., Hofer, H.W., and Wimmer, M. (2009). Cell and molecular biology of the novel protein tyrosine-phosphatase-interacting protein 51. International review of cell and molecular biology 275, 183-246.

Stingl, J., and Caldas, C. (2007). Molecular heterogeneity of breast carcinomas and the cancer stem cell hypothesis. Nat Rev Cancer 7, 791-799.

Stingl, J., Eirew, P., Ricketson, I., Shackleton, M., Vaillant, F., Choi, D., Li, H.I., and Eaves, C.J. (2006). Purification and unique properties of mammary epithelial stem cells. Nature 439, 993-997.

Sugimoto, Y., Whitman, M., Cantley, L.C., and Erikson, R.L. (1984). Evidence that the Rous sarcoma virus transforming gene product phosphorylates phosphatidylinositol and diacylglycerol. Proceedings of the National Academy of Sciences of the United States of America 81, 2117-2121.

Sun, Y., Fang, Y., Yoon, M.S., Zhang, C., Roccio, M., Zwartkruis, F.J., Armstrong, M., Brown, H.A., and Chen, J. (2008). Phospholipase D1 is an effector of Rheb in the mTOR pathway. Proceedings of the National Academy of Sciences of the United States of America 105, 8286-8291.

Takeishi, K., Taketomi, A., Shirabe, K., Toshima, T., Motomura, T., Ikegami, T., Yoshizumi, T., Sakane, F., and Maehara, Y. (2012). Diacylglycerol kinase alpha enhances hepatocellular carcinoma progression by activation of Ras-Raf-MEK-ERK pathway. Journal of hepatology.

Topham, M.K., Bunting, M., Zimmerman, G.A., McIntyre, T.M., Blackshear, P.J., and Prescott, S.M. (1998). Protein kinase C regulates the nuclear localization of diacylglycerol kinase-zeta. Nature 394, 697-700.

Toschi, A., Lee, E., Xu, L., Garcia, A., Gadir, N., and Foster, D.A. (2009). Regulation of mTORC1 and mTORC2 complex assembly by phosphatidic acid: competition with rapamycin. Molecular and cellular biology 29, 1411-1420.

Troussard, A.A., McDonald, P.C., Wederell, E.D., Mawji, N.M., Filipenko, N.R., Gelmon, K.A., Kucab, J.E., Dunn, S.E., Emerman, J.T., Bally, M.B., and Dedhar, S. (2006). Preferential dependence of breast cancer cells versus normal cells on integrin-linked kinase for protein kinase B/Akt activation and cell survival. Cancer Res 66, 393-403.

Urtreger, A.J., Kazanietz, M.G., and Bal de Kier Joffe, E.D. (2012). Contribution of individual PKC isoforms to breast cancer progression. IUBMB Life 64, 18-26.

van Blitterswijk, W.J., and Houssa, B. (2000). Properties and functions of diacylglycerol kinases. Cellular signalling 12, 595-605.

van den Bout, I., and Divecha, N. (2009). PIP5K-driven PtdIns(4,5)P2 synthesis: regulation and cellular functions. Journal of cell science 122, 3837-3850.

Vanhaesebroeck, B., Guillermet-Guibert, J., Graupera, M., and Bilanges, B. (2010). The emerging mechanisms of isoform-specific PI3K signalling. Nat Rev Mol Cell Biol 11, 329-341.

Verdeil, G., Chaix, J., Schmitt-Verhulst, A.M., and Auphan-Anezin, N. (2006). Temporal cross-talk between TCR and STAT signals for CD8 T cell effector differentiation. European journal of immunology 36, 3090-3100.

Verrier, E., Wang, L., Wadham, C., Albanese, N., Hahn, C., Gamble, J.R., Chatterjee, V.K., Vadas, M.A., and Xia, P. (2004). PPARgamma agonists ameliorate endothelial cell activation via inhibition of diacylglycerol-protein kinase C signaling pathway: role of diacylglycerol kinase. Circ Res 94, 1515-1522.

Veverka, V., Crabbe, T., Bird, I., Lennie, G., Muskett, F.W., Taylor, R.J., and Carr, M.D. (2008). Structural characterization of the interaction of mTOR with phosphatidic acid and a novel class of inhibitor: compelling evidence for a central role of the FRB domain in small molecule-mediated regulation of mTOR. Oncogene 27, 585-595.

Visvader, J.E. (2009). Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. Genes Dev 23, 2563-2577.

Walker, A.K., Jacobs, R.L., Watts, J.L., Rottiers, V., Jiang, K., Finnegan, D.M., Shioda, T., Hansen, M., Yang, F., Niebergall, L.J., Vance, D.E., Tzoneva, M., Hart, A.C., and Naar, A.M. (2011). A conserved SREBP-1/phosphatidylcholine feedback circuit regulates lipogenesis in metazoans. Cell 147, 840-852.

Weigelt, B., and Bissell, M.J. (2008). Unraveling the microenvironmental influences on the normal mammary gland and breast cancer. Seminars in cancer biology 18, 311-321.

Weigelt, B., Warne, P.H., and Downward, J. (2011). PIK3CA mutation, but not PTEN loss of function, determines the sensitivity of breast cancer cells to mTOR inhibitory drugs. Oncogene.

Whatmore, J., Wiedemann, C., Somerharju, P., Swigart, P., and Cockcroft, S. (1999). Resynthesis of phosphatidylinositol in permeabilized neutrophils following phospholipase Cbeta activation: transport of the intermediate, phosphatidic acid, from the plasma membrane to the endoplasmic reticulum for phosphatidylinositol resynthesis is not dependent on soluble lipid carriers or vesicular transport. The Biochemical journal 341 (Pt 2), 435-444.

Wickstrom, S.A., Lange, A., Montanez, E., and Fassler, R. (2010). The ILK/PINCH/parvin complex: the kinase is dead, long live the pseudokinase! The EMBO journal 29, 281-291.

Wiederschain, D., Wee, S., Chen, L., Loo, A., Yang, G., Huang, A., Chen, Y., Caponigro, G., Yao, Y.M., Lengauer, C., Sellers, W.R., and Benson, J.D. (2009). Single-vector inducible lentiviral RNAi system for oncology target validation. Cell Cycle 8, 498-504.

Wymann, M.P., and Schneiter, R. (2008). Lipid signalling in disease. Nat Rev Mol Cell Biol 9, 162-176.

Yanagisawa, K., Yasuda, S., Kai, M., Imai, S., Yamada, K., Yamashita, T., Jimbow, K., Kanoh, H., and Sakane, F. (2007). Diacylglycerol kinase alpha suppresses tumor necrosis factor-alpha-induced apoptosis of human melanoma cells through NF-kappaB activation. Biochimica et biophysica acta 1771, 462-474.

Yeaman, C., Ayala, M.I., Wright, J.R., Bard, F., Bossard, C., Ang, A., Maeda, Y., Seufferlein, T., Mellman, I., Nelson, W.J., and Malhotra, V. (2004). Protein kinase D

regulates basolateral membrane protein exit from trans-Golgi network. Nature cell biology 6, 106-112.

Zhang, J., Liu, X., Datta, A., Govindarajan, K., Tam, W.L., Han, J., George, J., Wong, C., Ramnarayanan, K., Phua, T.Y., Leong, W.Y., Chan, Y.S., Palanisamy, N., Liu, E.T., Karuturi, K.M., Lim, B., and Miller, L.D. (2009). RCP is a human breast cancer-promoting gene with Ras-activating function. The Journal of clinical investigation 119, 2171-2183.

Zhao, C., Du, G., Skowronek, K., Frohman, M.A., and Bar-Sagi, D. (2007). Phospholipase D2-generated phosphatidic acid couples EGFR stimulation to Ras activation by Sos. Nature cell biology 9, 706-712.

Zhong, X.P., Hainey, E.A., Olenchock, B.A., Jordan, M.S., Maltzman, J.S., Nichols, K.E., Shen, H., and Koretzky, G.A. (2003). Enhanced T cell responses due to diacylglycerol kinase zeta deficiency. Nature immunology 4, 882-890.

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APPENDIX

ARTICLES PUBLISHED

1. Isabel Mérida, Antonia Ávila-Flores, Job García, Ernesto Merino, María Almena, **Pedro Torres-Ayuso** (2009). Diacylglycerol kinase alpha, from negative modulation of T cell activation to control of cancer progression. ***Adv. Enz. Regul.*** 49:174-188

2. Severine I. Gharbi, Esther Rincón, Antonia Ávila-Flores, **Pedro Torres-Ayuso**, María Almena, María Ángeles Cobos, Juan Pablo Albar, Isabel Mérida (2011). Diacylglycerol kinase ζ controls diacylglycerol metabolism at the immunological synapse. ***Mol. Biol. Cell.*** 22:4406-4414

3. Mónica Martínez-Moreno, Job García-Liévana, Denise Soutar, **Pedro Torres-Ayuso**, Elena Andrada, Xiao-Ping Zhong, Gary A. Koretzky, Isabel Mérida, Antonia Ávila-Flores. FoxO-dependent regulation of diacylglycerol kinase α gene expression. ***Manuscript under revision.***

4. **Pedro Torres-Ayuso**, Isabel Mérida, Antonia Ávila-Flores. Diacyglycerol consumption by DGKζ contributes to the maintenance of the lipogenic metabolism of breast tumors. ***Manuscript in preparation***

5. **Pedro Torres-Ayuso**, David R. Jones, Nullin Divecha, Isabel Mérida, Antonia Ávila-Flores. Essential role of diacylglycerol kinase activity for PI3K/AKT/mTOR signaling in breast cancer cells. ***Manuscript in preparation.***